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14. ABSTRACT
The overall goal of this work is to develop ultra-sensitive detection techniques to identify a panel of new biomarkers and indicators with diagnostic and predictive value in breast cancer. During year 1, we identified candidate breast cancer biomarkers and developed ultra-sensitive assays for several of them. Two different miRNA single molecule assay approaches have also been investigated and both showed promise for achieving similar ultrasensitive data using miRNA as targets. Continuing work on this assay includes increasing efficiency and decreasing the background. Circulating tumor cell isolation using microfluidics has been accomplished and these methods will soon be integrated with the single molecule detection approach. Good progress on identifying additional markers (miRNA, mtDNA, ADAM8) has been made. Further work to validate whether these markers are useful for detecting early stage breast cancer or tumor aggressiveness is underway. Finally, we have begun testing serum collected from a Human-in-Mouse (HIM) model of breast cancer using the assays developed.

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Statement of Work

The overall goal of this work is to develop ultra-sensitive detection techniques in order to identify a panel of new biomarkers and indicators with diagnostic and predictive value in breast cancer. During years 1 and 2 we will develop ultra-sensitive detection techniques and apply them to identifying prospective biomarkers using the Human-in-Mouse (HIM) model of breast cancer. During years 3-5 we will extend the findings in the HIM model to validate prospective biomarkers in human subjects with breast cancer. This work is broken down into specific tasks by investigator as follows:

INTRODUCTION

Despite the recent advances made in breast cancer diagnostics and treatment, it has been estimated that in 2012 the United States will diagnose approximately 200,000 new cases of breast cancer, resulting in approximately 40,000 deaths.[1] Mammography is a powerful imaging technique for tumor detection; however, it lacks the ability to decipher benign from cancerous tumors, is unable to detect tumors smaller than 1mm,[2] misses approximately 20% of breast cancers potentially present at the time of screening, and has an 8-10% false positive rate.[3] These drawbacks lead to inaccurate patient diagnosis, which can allow potentially fatal disease progression, or in the cases of over-treatment, unnecessary physical and emotional trauma.[4] ELISA, the most common immunoassay for measuring proteins from breast tumors, excised samples, and serum, has a lower detection limit of ~1-10 pM,[5] which is not sensitive enough to measure low abundance proteins, RNAs, and other biomarkers that could aid in the early and reliable diagnosis of cancer. There is a strikingly clear need to develop techniques capable of detecting biomarkers specific for breast cancer that will enable earlier diagnosis of disease, prediction of patient outcome, and improve therapeutic efficacy in a non-invasive manner. Our goals are to utilize ultrasensitive single molecule techniques developed in our laboratory to discover new biomarkers that meet these requirements within serum so that a simple blood test can be implemented. We are also working to characterize breast cancer biopsy samples with single cell resolution to discover the nature of the underlying heterogeneity in complex cell populations with the goal of correlating disease outcome with genotypes and phenotypes of individual cells.

BODY

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Task 1. Develop single molecule diagnostics using HIM (Human-in-Mouse) model (Years 1-2).

1a. Select approximately ten candidate markers (mt DNA, proteins, stem cell markers, etc.) in conjunction with collaborators (Months 1-6).

Upon discussion with Drs. Charlotte Kuperwasser and Rachel Buchsbaum, the selection of potential biomarkers comprised the following proteins based on their association with breast cancer progression: EpCAM, SPARC, SDF-1, IGFP-5, Vimentin, BMP-2, BMP-4, OPN, Tenascin-C, PVRL-4 (nectin 4), CD44 v6, SLUG, SNAIL, GATA-3, FGF-9, SERPIN-E2, and CD49f. Through literature searches as well as some testing, it was found that standard ELISA methods were already capable of detecting EpCAM[6], SPARC[7], SDF-1[8], IGFP-5[9], Vimentin[10], BMP-2 and BMP-4[11], OPN[12], Tenascin-C[13], and CD44 v6[14] in serum, meaning the development of a sensitive digital ELISA is not required for these proteins. Digital ELISA, however, can be useful for detecting the serum levels of SLUG, SNAIL, GATA-3, FGF-9, SERPIN-E2, and CD49f since no serum studies of these markers have been reported.

Also, several miRNAs were chosen for investigation as biomarkers for this study, including miRNA-21, miRNA-155, miRNA-145, miRNA-10b, and miRNA-125. All of these miRNAs have been identified to display aberrant expression in breast cancer. miRNA-21 is often over-expressed in tumors of several cancer types, including breast cancer, and has been associated with apoptosis and tumor progression.[15,16] miRNA-145 normally acts as a tumor suppressor by repressing c-Myc; however, in

cancer this miRNA is often underexpressed, leading to cell invasion and metastasis.[17,18] miRNA-10b has been found to target the mRNA HOXD10, a transcriptional repressor associated with cell migration and extracellular matrix remodeling. Deregulation of miRNA-10b has been related with tumor metastasis.[19,20] Finally, miRNA-155 is over-expressed in invasive breast cancers and has been associated with the TGF-β pathway, which has been found to play a critical role in breast cancer metastasis.[21]

We are continuing to expand this list by mining recent literature reports and discussing various markers with experts in the field.

1b. Develop single molecule assays for the candidate markers selected in 1a. Assays will be developed on microspheres and tested first on standard samples. Assay performance characteristics will be determined to ascertain that they will address concentration ranges of interest and requisite precision. For general protocols see: Nature Biotechnology, 2010, 28, 595-599 (Months 3-15).

Digital ELISA Development

EpCAM (epithelial cell adhesion molecule) is a cell surface molecule that plays an important role in cell adhesion and signaling.[17] Normal expression of EpCAM in serum as measured by ELISA is approximately 16 pM, 640 pg/mL.[6] In cancer patients, EpCAM is over-expressed and has been measured in serum using ELISA at levels greater than 50 pM, 2 ng/mL.[6,22] With this information, it was hypothesized that EpCAM had the potential to be a good validating marker to determine the feasibility of digital ELISA for measuring tumor progression in serum obtained from the HIM model terminal bleeds (*see* Task 1c for HIM model description). As a result, an assay was first developed for measuring human EpCAM using digital ELISA.

Antibody pair compatibility

Initial experiments for developing a digital ELISA assay for EpCAM involved choosing appropriate antibody pairs to recognize EpCAM in a sandwich format as well as optimizing the conjugation efficiency of selected capture antibodies onto magnetic microspheres. First, a standard sandwich ELISA was performed to analyze antibody compatibility to optimize for the digital ELISA. **Figure 1** illustrates the assay response for the selected monoclonal (capture) and polyclonal (detection) antibody pair of interest (R & D Systems) using a sandwich ELISA with horseradish peroxidase (HRP) as the enzymatic reporter. Increasing ELISA response (in absorbance units) correlated well with increased concentrations of human EpCAM protein standard.

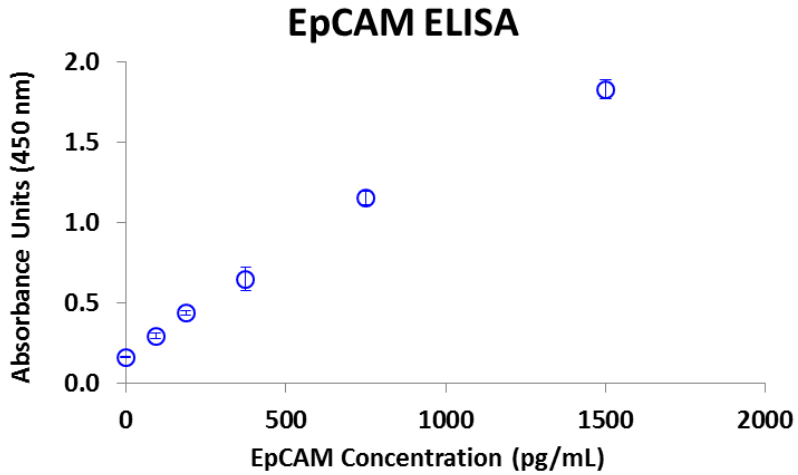


Figure 1: Sandwich ELISA analysis of monoclonal and polyclonal antibody pair recognizing human EpCAM (R&D Systems)

Antibody-microsphere conjugation efficiency

The monoclonal capture antibodies (R&D Systems) acting against EpCAM were attached to 2.7 µm carboxyl-terminated magnetic microspheres (Agilent). The coupling was performed using conjugation chemistry with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC)[23] (Thermo) to create a covalent bond between the microsphere and the antibody. Different concentrations of EDC (0.1 mg/mL and 0.5 mg/mL) were used to determine sufficient antibody coverage onto the microspheres. The antibody-coated microspheres were then evaluated in a bulk sandwich ELISA using β-galactosidase as the reporter to determine which batch yielded the highest response for measuring EpCAM. **Figure 2** shows the results from this immunoassay and demonstrates that 0.5mg/mL of EDC provided the most optimal capture antibody coverage.

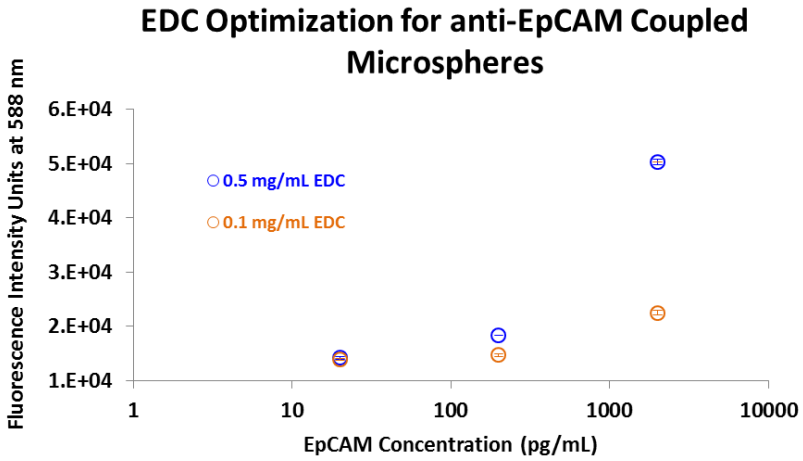


Figure 2: Assay response for antibody-coupled microspheres targeting EpCAM using 0.5 mg/mL and 0.1 mg/mL EDC.

Next, the selected antibody-conjugated microspheres (prepared using 0.5 mg/mL EDC) were used for digital ELISA for sensitive detection of EpCAM. **Figure 3** shows that a linear response between 0 and 10 pg/mL (0 to 250 fM) was achieved with a limit of detection of 0.88 pg/mL (22 fM). The associated coefficient of variation (CV) ranged between 4 to 16%. This assay was then extended to measuring serum samples obtained from terminal bleeds of the HIM models containing tumors grown from EpCAM-positive cell lines. More details of this work will be discussed in Task 1c. This assay was 100 times more sensitive than the conventional ELISA, attesting to the potential value of the digital ELISA.

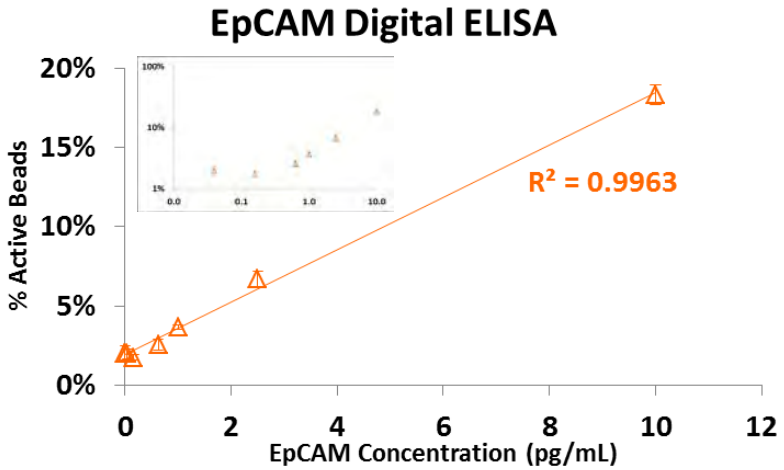


Figure 3: Dose-response curve using digital ELISA for measuring EpCAM with a calculated LOD of 0.88 pg/mL (22 fM). Inset shows a log-log plot of the same data.

Extension to biomarkers with potential diagnostic application

Efforts were also made to develop assays for biomarkers that would be implemented for diagnostic applications (GATA3, SERPIN-E2, and FGF-9). Assay development for FGF-9 was accomplished in a similar approach to EpCAM and began with evaluation of antibody pair compatibility. FGF-9 (Fibroblast growth factor) is involved in morphogenesis, angiogenesis, and tissue remodeling, and is up-regulated in breast cancer.[24,25] Using a selected pair of antibodies recognizing FGF-9 (R&D Systems), the compatibility of the antibodies for detecting FGF-9 was evaluated by performing a sandwich ELISA. **Figure 4** shows the assay response using ELISA illustrating the correlation between increasing optical signal with increasing concentrations of FGF-9. Here, β-galactosidase was used as the enzymatic reporter.

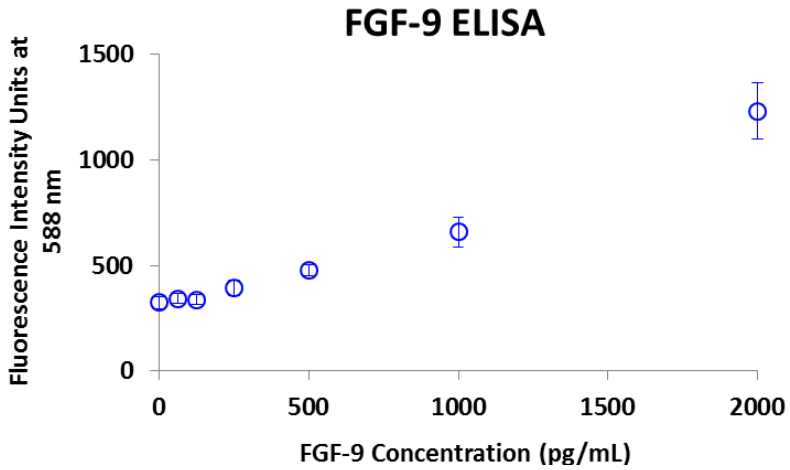


Figure 4: Sandwich ELISA analysis of monoclonal and polyclonal antibody pair recognizing FGF-9 (R&D Systems).

Reagent concentrations used for antibody-microsphere conjugation were then optimized using the selected capture antibody. **Figure 5** shows the bulk sandwich immunoassay response using the antibody-conjugated microspheres prepared with different EDC concentrations with β-galactosidase as the reporter. From this experiment it was found that 0.5 mg/mL of EDC was the optimal amount of reagent necessary for efficient antibody conjugation onto the microspheres.

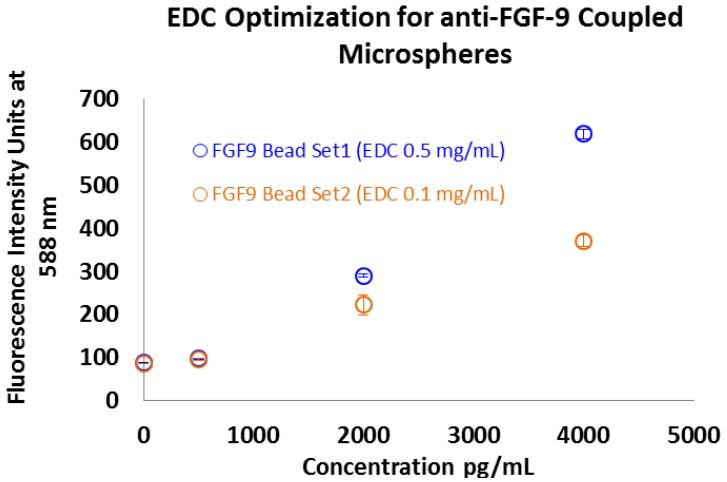


Figure 5: Assay response for antibody-coupled microspheres targeting FGF-9 using 0.5 mg/mL and 0.1 mg/mL EDC. The optimal amount of reagent necessary for efficient antibody conjugation onto the microspheres was 0.5 mg/mL of EDC.

The selected antibody-conjugated microspheres (prepared using 0.5 mg/mL EDC) were then used for digital ELISA for sensitive detection of FGF-9. It was found that at dilute concentrations (i.e. below

20 pg/mL) where the sensitivity of digital ELISA would be advantageous, no response to increasing concentrations of FGF-9 protein standard above background was observed (**Figure 6**).

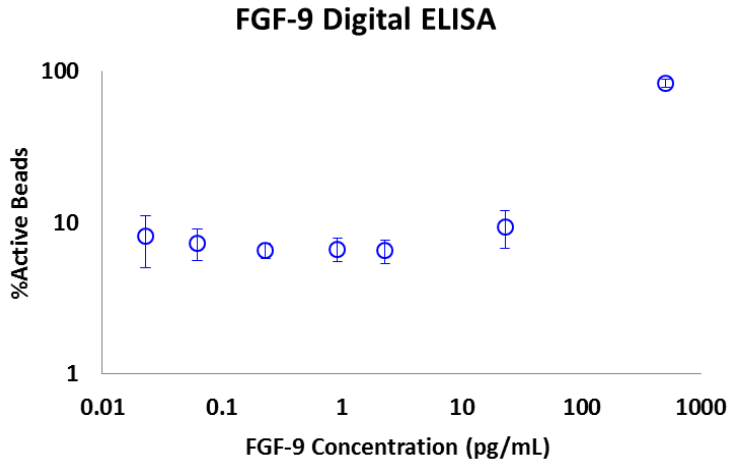


Figure 6: Dose-response curve using digital ELISA for measuring FGF-9. No increase in signal is present below 20 pg/mL.

The translation of the FGF-9 assay from bulk measurements to digital ELISA was not successful and we speculate that the lack of sensitivity is due to the binding affinity of the selected antibody pair. It is possible that at higher concentrations (within that of the range of ELISA) the antibody pair may have sufficient binding affinity to detect FGF-9 using the ELISA format. At low concentrations that are within the range for digital ELISA, the binding affinities of these antibodies may either not be sufficiently strong to capture all present proteins in solution to give rise to their detection. Similarly, it is possible that the off rate is too fast for the captured protein to be retained on the microsphere during the washing steps.

GATA-3 and SERPIN-E2

Monoclonal and polyclonal antibodies recognizing human SERPIN-E2 and GATA-3 were purchased from various manufacturers (Santa Cruz Biotechnology, Abnova, Abcam, R&D Systems). These antibodies were then evaluated in a sandwich format using ELISA to determine their compatibility for measuring increasing concentrations of their associated antigens. Preliminary results from screening experiments have resulted in ELISA responses at background levels for increasing concentration of protein. These results suggest that the antibodies under evaluation were not suited for sandwich-type recognition of their corresponding proteins.

Due to incompatible antibody pairs and insufficient assay response of chosen capture antibodies attached to microspheres, much work still needs to be done to develop and optimize assays for measuring FGF-9, GATA-3, and SERPIN-E2 for further application to digital ELISA. Future approaches will include a more comprehensive screening of available antibodies from other manufacturers as well as investigating other useful biomarkers for breast cancer detection in serum.

miRNA SiMoA Development

The pre-established protocol for SiMoA assays applies well for protein assays;[26] however, since this platform has not been used for miRNA assays before, it requires modification and optimization for use with miRNA biomarkers. Unlike several of the common detection methods for miRNAs, such as RT-PCR, our proposed methods do not require target amplification, eliminating potential bias and offering a digital readout at the single molecule level using enzymatic signal amplification. miRNAs are challenging molecules to target for single molecule assays due to their short length, secondary structures, and multiple isomers. To aid in overcoming these difficulties, two separate approaches are currently being implemented, ligation miRNA SiMoA and sandwich miRNA SiMoA.

Ligation miRNA SiMoA

Ligation miRNA SiMoA utilizes a three step ligation reaction where T4 RNA polymerase is used to ligate miRNAs in a target solution to 5'phosphorylated DNA (cp-Ligate) covalently attached to magnetic microspheres (**Figure 7**). Once the ligation process is complete, a biotinylated complementary signal DNA probe will hybridize to the ligated miRNA-DNA complex on the microsphere. Finally, streptavidin-β-galactosidase is introduced and will bind to the complex via biotin-streptavidin binding. The microspheres are then loaded onto a 50,000 well fiber array. In the presence of the substrate resorufin β-D-galactopyranoside, a fluorescent product will be produced for each microsphere containing a single molecule of the target miRNA. Although ligation using T4 RNA ligase is typically used for cloning purposes, a similar approach was recently used for non-single molecule ssRNA detection using SPR.[27]

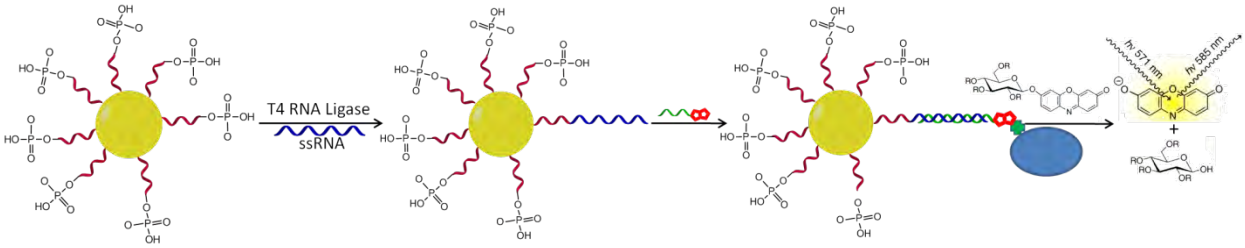
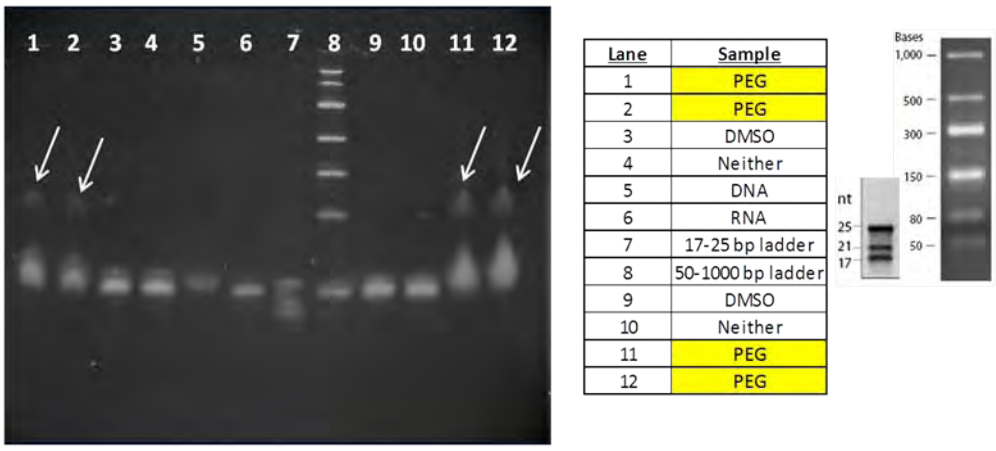


Figure 7: Scheme of single molecule miRNA ligation on microsphere. Magnetic microspheres with 5'phosphorylated DNA covalently attached are incubated with T4 RNA ligase and miRNA target. The 3' hydroxylated end of miRNA ligates to DNA on the microsphere creating a DNA-RNA hybrid oligo via a phosphodiester bond. A biotinylated DNA signal probe is hybridized to the DNA-RNA hybrid and streptavidin-β-galactosidase is introduced and bound. In the presence of resorufin β-D-galactopyranoside, a flurorescent signal is produced.

Validation of Ligation Reaction

In order to validate the ligation process and to help determine practical reaction conditions, the assay was first performed in bulk solution. **Figure 8**, shown below, demonstrates the successful ligation of miRNA-21 with the intended 5'phosphorylated DNA target. Multiple reaction conditions were tested, including different concentrations of PEG 8000 and DMSO, combinations of both of these reagents, and the absence of both. The gel below shows an experiment where successful ligation was found to occur optimally in the presence of 25% PEG solution. It has been noted that the addition of 12.5 to 25% PEG 8000 stimulates ligation in bulk solution by creating macromolecular crowding.[28] The addition of 10% DMSO has also been shown in the literature to enhance ligation by decreasing the secondary structures of RNA that may shield the ends and inhibit ligation[29]; however, the addition of DMSO did not seem to have any positive effects on ligation in these experiments.



Lane	Sample
1	PEG
2	PEG
3	DMSO
4	Neither
5	DNA
6	RNA
7	17-25 bp ladder
8	50-1000 bp ladder
9	DMSO
10	Neither
11	PEG
12	PEG

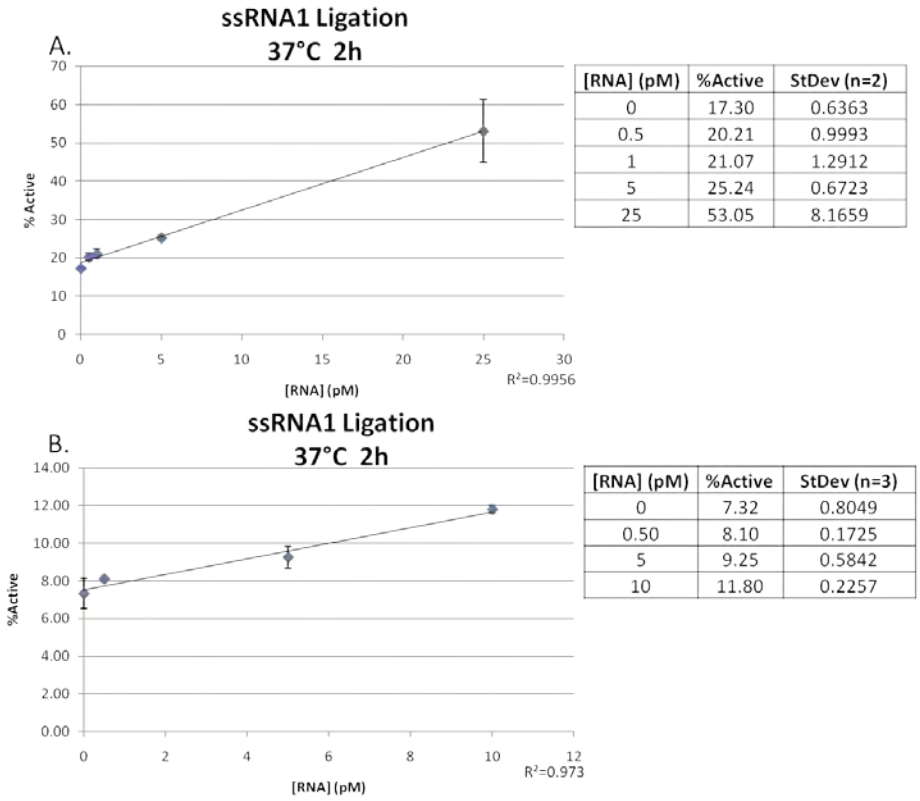
Figure 8: 15% TBE Urea gel with SYBR gold stain showing successful bulk ligation of miRNA-21 (22 bp). Each band has 0.2 μg sample, which consisted of a 50 μL reaction with 2 μL of 100 μM RNA and 2 μL DNA (5'Phos$T_{24,}$ 24 bp). Samples had

either 25% PEG 8000, 10% DMSO, or neither as indicated in the table. Lane 8 has contamination from lane 9 (from loading gel). Arrows point out that the lanes with 25% PEG are the only ones showing successful ligation product (46 bp). Ladders on right are the 17-25 bp and 50-1000 bp ladders run in lanes 7 and 8.

Despite the positive effects that the addition of PEG 8000 had on the success of ligation in bulk reactions, when a similar experiment was attempted at the single molecule level, virtually no signal was obtained (data not shown). These results can be explained because in bulk, the primary function of the PEG is to create macromolecular crowding, which leads to high effective concentrations of substrate termini.[28] Single molecule reactions occur at significantly lower concentrations, so it is likely that the concentration of miRNA is too low for efficient reaction. It is also possible that when PEG is introduced, it may create too much crowding and reduces the probability that the ligation reaction of RNA target to the DNA on the microsphere surface. The PEG may also simply be coating the microspheres, effectively blocking ligation.

Preliminary single molecule experiments were performed using a 24 bp RNA sequence that will be abbreviated here as ssRNA1. The capture probe for these experiments is universal and remains the same for all ligation experiments. The signal probe however, is specific (sp-ssRNA). All sequences are listed in the Supporting Data under **Table S1**. This sequence was chosen because it has been shown in previous work to have limited secondary structure and thus will function as a miRNA standard for assay development.[27] Once the assay is proven, the miRNAs chosen as biomarkers will be used and optimization will be required for each biomarker.

The first single molecule ligation experiments were performed at 37°C for two hours, as recommended by the manufacturer of the ligase (Promega). **Figure 9A** shows a calibration curve of the ligation of ssRNA1 from 0.5 pM to 25 pM. The assay showed good reproducibility at low concentrations. The higher concentration range showed more variability, but the overall calculated LOD for this assay ~350 fM (~2.1 $x10^7$ molecules). The assay was repeated, as shown in **Figure 9B**, with the same assay conditions, but with a different set of DNA coupled microspheres. The data for this assay shows a lower background but has lower overall signal response, resulting in a higher LOD of ~5.5 pM (~$3.3x10^8$ molecules). The discrepancy between these two experiments was also demonstrated among other experiments and is thought to be due partly from the process of covalently attaching DNA to the magnetic microspheres. In order to assess potential causes for the assay irreproducibility and high background, several different parameters were investigated, including ligation temperature, time, and capture probe type.



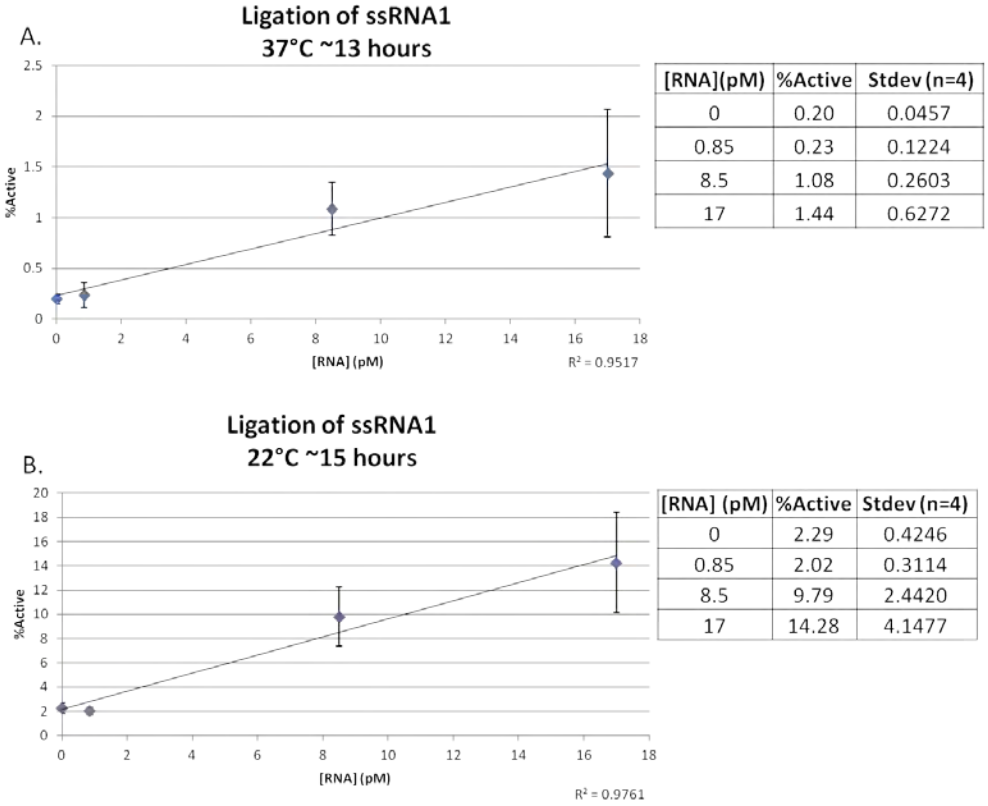
[RNA] (pM)	%Active	StDev (n=2)
0	17.30	0.6363
0.5	20.21	0.9993
1	21.07	1.2912
5	25.24	0.6723
25	53.05	8.1659

[RNA] (pM)	%Active	StDev (n=3)
0	7.32	0.8049
0.50	8.10	0.1725
5	9.25	0.5842
10	11.80	0.2257

Figure 9: Calibration curves for the ligation of ssRNA1 to microspheres for 2 hours at 37°C. The tables on the graph insets show the corresponding %Active and standard deviation values. The calculated LODs for each curve were A.) ~350 fM and B.) ~5.5 pM. Data for B was collected approximately one month after A and with a different set of microspheres.

Optimizing Ligation Reaction Conditions

The manufacturer of the ligase (Promega) states that the ligation reaction can occur at 37°C for shorter periods of time or at room temperature overnight.[30] Ligation reactions using ssRNA1 were performed at 37°C and at 22°C overnight (13-15 hours). **Figure 10A** and **B** show representative results from experiments performed under these conditions. The background in both assays was much lower than in the previous two experiments, but the same large standard deviation is displayed at higher concentration levels. Both temperature experiments had similar LODs, ~1.3 pM ($7.8x10^7$ molecules) and ~1.9 pM ($1.1x10^8$ molecules), respectively for 37°C and at 22°C, but the response from the 22°C curve appears to be more sensitive, yielding higher %Active values for the corresponding concentrations.



[RNA](pM)	%Active	Stdev (n=4)
0	0.20	0.0457
0.85	0.23	0.1224
8.5	1.08	0.2603
17	1.44	0.6272

[RNA] (pM)	%Active	Stdev (n=4)
0	2.29	0.4246
0.85	2.02	0.3114
8.5	9.79	2.4420
17	14.28	4.1477

Figure 10: Calibration curves for ssRNA1 overnight at 37°C (A) and 22°C (B) with calculated LODs of ~1.3 pM ($7.8x10^7$ molecules) and LOD: ~1.9 pM ($1.1x10^8$ molecules), respectively. The tables in the graph insets show the corresponding %Actives and standard deviation values.

Testing of Different Capture Probes

Different capture probes were investigated by coupling DNA complementary to the ssRNA sequence (cp-DNA), as well as a DNA sequence with an internal PEG spacer (cp-PEG), and a locked nucleic acid (LNA) sequence (cp-LNA) to separate sets of microspheres. To test the microspheres, direct hybridization experiments were performed using the same ssRNA with the addition of a 5' biotin tag (b-RNA). **Figure 11A-C** show the resulting calibration curves from experiments performed with these three microsphere types from 10 fM–1 pM. All microspheres were coupled on the same day and assays were performed on the same day, approximately 3 weeks after coupling. The results show that when biotinylated RNA was allowed to hybridize for 2 hours at 37°C, which are the ligation assay conditions, the microspheres with DNA displayed very high background signals. Microspheres coupled with DNA containing a PEG spacer had a significantly lower background signal and yielded the lowest LOD (2.8 fM). Microspheres coupled with LNAs also had low background signal, but the overall signal response was low. ***The ability to detect fM concentration levels for miRNAs is unprecedented so we are hopeful that this method will now be useful for breast cancer related miRNAs.***

From these experiments it is apparent that adding the PEG spacer to the capture probe was efficient in decreasing the background signal. This result can be explained because the majority of background signal is caused by non-specific binding of either target or enzyme. PEG is a common blocking agent and the PEG spacers in the capture probes most likely help to block any unwanted binding events. Further optimization can be performed using other PEG spacers of varying lengths.

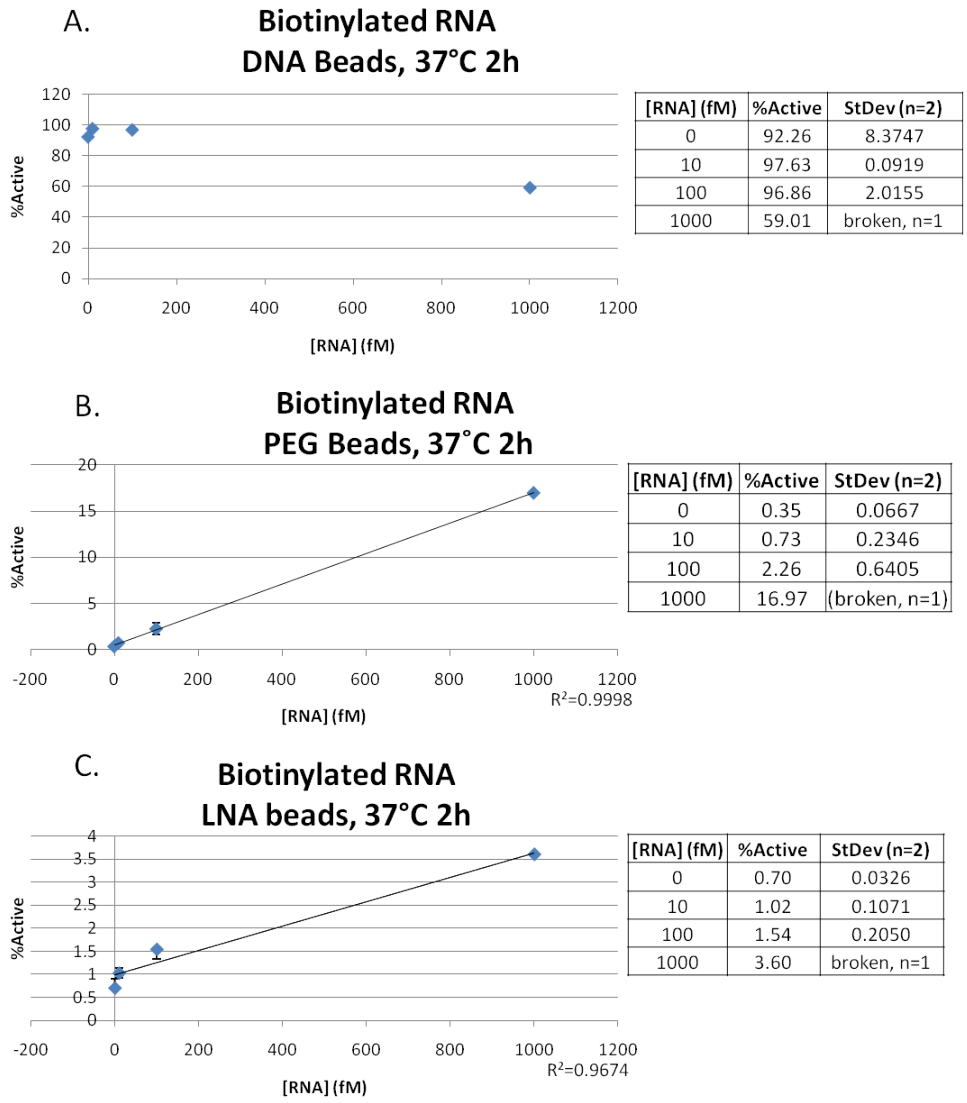


Figure 11: Calibration curves for direct hybridization experiments with microspheres coupled with different capture probes (A) DNA (B) DNA with an internal PEG spacer, and (C) LNA. The calculated LOD for the PEG and LNA microspheres were 2.8 and 10 fM, respectively. The tables in the graph insets show the corresponding %Actives and standard deviation values.

The overall efficiency of the ligation assay is low, as demonstrated by the high LODs and low %Actives in the single molecule assays. This result could be due to the additional enzymatic reaction required for this assay. As previously mentioned, the ligation process consists of three steps. The first step involves adenylation of a lysine residue at the active site on the T4 RNA ligase. Next, adenosine monophosphate (AMP) is transferred to the 5'phosphorylated nucleic acid donor (in this case the DNA on the magnetic microspheres). Finally, the formation of the phosphodiester bond between the 5'DNA donor and the 3'hydroxylated acceptor nucleic acid (in this case the miRNA) is promoted by the ligase. The fact that this entire procedure requires an additional enzymatic reaction¸ especially a three step process, may definitely contribute to the restrictions we seem to be facing in the lower detection limit range.

Although there are limitations associated with the ligation approach to detecting miRNA, the advantage is that with ligation the entire miRNA sequence is available for binding to the signal probe. This approach allows for an increase in specificity despite potential loss in sensitivity due to the effects of the additional enzymatic step. Since there are many miRNAs with several isoforms that differ by only a

single base pair yet have different biological implications, target specificity is of vital importance for this assay and for the development of any miRNA assay. Further assay optimization is required to enhance sensitivity and reproducibility.

Sandwich miRNA SiMoA

The second approach for miRNA detection is a sandwich hybridization assay, depicted in **Figure 12**. In this approach, microspheres are conjugated with a DNA capture probe complementary to half of the target miRNA sequence of interest. The DNA coated microspheres are incubated in solution with both the target miRNA and a complementary biotinylated DNA signal probe, creating a sandwich. The sandwich hybridization complex is then incubated with streptavidin-β-galactosidase and in the presence of substrate, measurable fluorescence is generated.

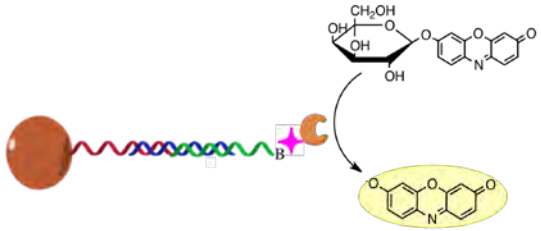


Figure 12: Schematic of miRNA sandwich hybridization assay. The DNA capture probe (red) is incubated with the target sequence (blue) and biotinylated DNA signal probe (green). Once streptavidin-β-galactosidase (pink/brown) is bound to the signal probe, the complex is incubated with substrate, generating fluorescent resorufin.

Microsphere Conjugation Experiments

Initial experiments for this approach assessed microsphere conjugation, which is vital for target capture and obtaining reproducible signal. These assays were performed in bulk using a microtiter plate reader measuring fluorescence. For initial experiments, the DNA analog of the miRNA target (miRNA-21 DNA) was used to eliminate the possibility of RNA degradation due to RNase contamination. The capture and signal probes used in this assay are listed in **Table S1** as cp-miRNA-21 and sp-miRNA-21, respectively. **Figure 13** shows a sandwich hybridization assay in bulk solution from 5-50pM of DNA target with a LOD of 1.8 pM. The data collected from this and other experiments show the successful conjugation of DNA probes to the microspheres.

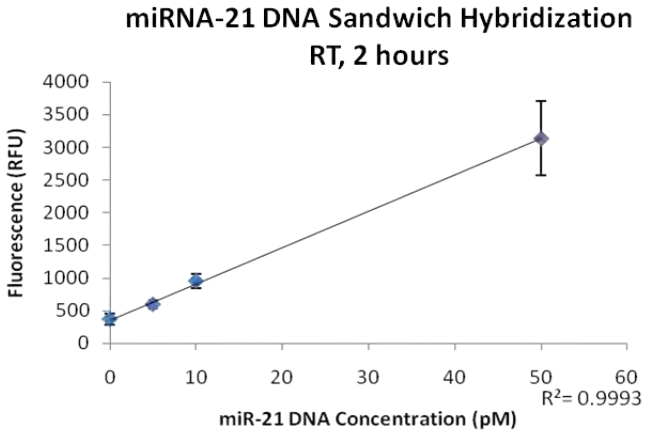


Figure 13: Sandwich hybridization bulk assay showing fluorescence generated at concentrations in the picomolar range. Microspheres were allowed to hybridize for 2 hours, followed by a 30 minute incubation with 50 pM enzyme, and a one hour incubation with 33 mM substrate before measurement. The calculated LOD for this experiment was 1.8 pM.

Once a working assay was achieved at the bulk level, optimization was required for the single molecule assay. Concentrations of biotinylated signal probe and enzyme were adjusted accordingly, and the incubation with substrate was performed with the microspheres in the wells etched into fiber optic

bundles. There have been a number of challenges associated with optimizing this assay for low femtomolar concentrations of target, including high background, unexpectedly low signal, and overall inconsistency between replicates. **Figure 14** gives an example of a miRNA-21 DNA analog assay at the single molecule level displaying some of the above challenges.

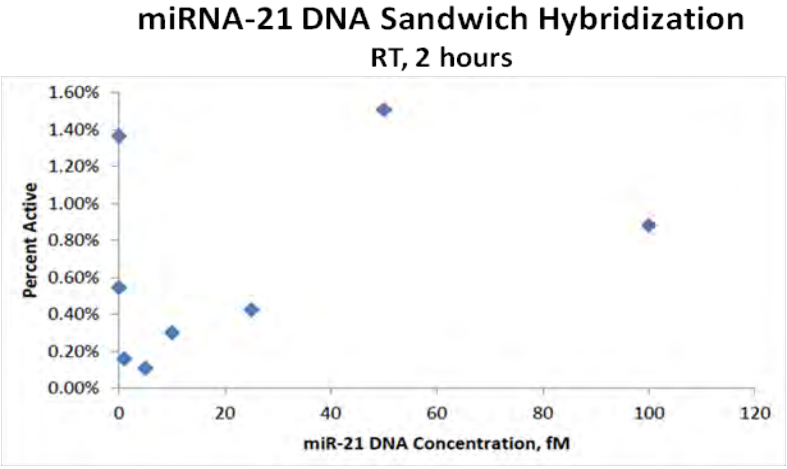
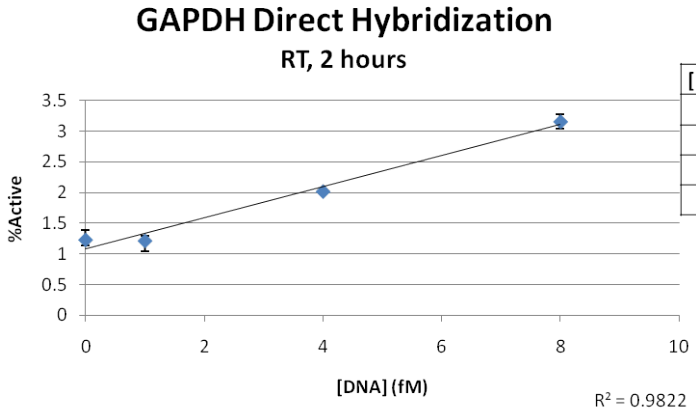


Figure 14: Sandwich hybridization assay showing percent of active microspheres over a range of concentrations in the femtomolar region. The overall signal generated is extremely low for the concentrations of target in solution, and the background is high relative to the rest of the samples. This graph is an example of one of the challenges faced in single molecule assay optimization.

Optimization of Hybridization Conditions

There are a variety of ways to optimize this assay to improve assay response, including altering temperature, hybridization time, modifying washes, buffers, and enzyme concentration etc. For some optimization experiments, assays were performed using a 20 bp DNA excerpt from the housekeeping gene GAPDH. **Figure 15** shows a calibration curve established at the single molecule level for a direct hybridization of the 20 bp biotinylated GAPDH probe (DH-GAPDH). Although the assay response is slightly lower than expected, meaning the assay is not as efficient as possible, the standard deviations are acceptably low and the calculated LOD was approximately 4 fM.

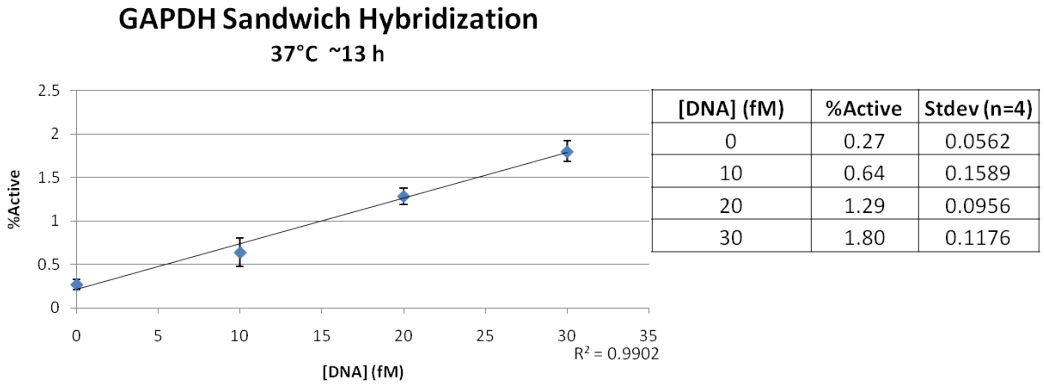


[DNA] fM	%Active	StDev %Active (n=2)
0	1.22	0.1594
1	1.21	0.0819
4	2.02	0.0354
8	3.16	0.1144

Figure 15: Calibration curve for direct hybridization of 23 bp GAPDH probe to magnetic microspheres. Assay was performed at room temperature for a total of 2 hours incubation time with target. Calculated LOD was ~4 fM. The table in the graph inset shows the corresponding %Actives and standard deviation values.

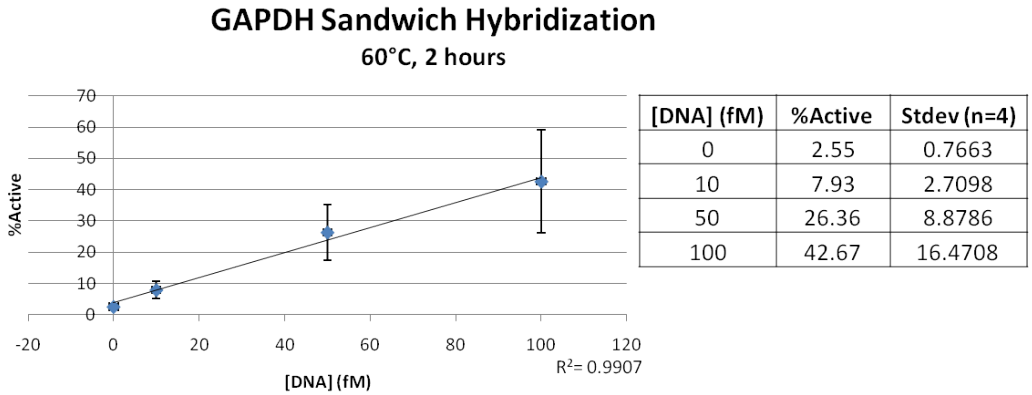
Once the direct hybridization experiments showed the expected response, sandwich DNA experiments with a slightly longer (65 bp) DNA probe (GAPDH-sand) were performed. Since initial attempts to perform sandwich or direct hybridization assays with the shorter miRNA-DNA analog probes were unsuccessful, several sandwich assays using this probe were attempted. **Figure 16** shows a

representative sandwich assay with the GAPDH target performed at 37°C for approximately 13 hours. Although the assay response was low, the reproducibility between replicates was good and the calculated LOD from four replicates was approximately 4.3 fM, comparable to the direct hybridization experiment. When a similar experiment was performed for only 2 hours at an increased temperature, 60°C, the LOD was even lower at 2.5 fM and had a higher response (**Figure 17**). More optimization needs to be performed on these assays to determine the most ideal conditions; however, increasing the hybridization temperature seems to have a slightly stronger impact on the LOD and assay efficiency than increasing the hybridization time. These two points will be taken into account for further assay development experiments.



[DNA] (fM)	%Active	Stdev (n=4)
0	0.27	0.0562
10	0.64	0.1589
20	1.29	0.0956
30	1.80	0.1176

Figure 16: Calibration curve for sandwich hybridization of GAPDH probe at 37°C for ~13 hours. The calculated LOD was ~4.3 fM. The table in the graph inset shows the corresponding %Actives and standard deviation values.



[DNA] (fM)	%Active	Stdev (n=4)
0	2.55	0.7663
10	7.93	2.7098
50	26.36	8.8786
100	42.67	16.4708

Figure 17: Calibration curve for sandwich hybridization of GAPDH probe at 60°C for 2 hours. The calculated LOD was ~2.5 fM. The table in the graph inset shows the corresponding %Actives and standard deviation values.

The above single molecule experiments are evidence that this assay is functioning moderately well with DNA and is able to detect low numbers of target DNA molecules. Future work for this assay will include using smaller targets for DNA and RNA sandwich assays and optimizing with different hybridization conditions for each target miRNA to decrease secondary structures that could inhibit binding and decrease assay efficiency. We will also investigate miRNAs with similar isoforms to test the specificity of the assay design and later RNA extractions will be explored to optimize sample preparation of biological fluids for single-molecule miRNA assays.

1c. Screen blood samples from 'Human-In-Mouse' (HIM) Model obtained from Kuperwasser (Months 12-24). HIM tumors will be created from human breast epithelial cells collected from discarded tissues of women who have undergone reduction mammoplasty surgeries. For more information/details on model: *Nature Protocols*, 2006, 1, 595-599.

In the past year, the Kuperwasser Laboratory created orthotopic humanized tissue-transgenic breast cancers using mammary epithelial cells obtained from reduction mammoplasty tissues from patients undergoing elective surgery. The creation of HIM tumors involves three distinct temporal steps: (1) clearing of the murine mammary fat pad, (2) reconstitution of the mammary fat pad with human stromal cells and (3) introduction of lentiviral-infected mammary epithelial cells co-mixed with activated fibroblasts into the humanized fat pad. For task 1, based on the biomarkers that were being assessed, we generated HIM tumors (N=10) using SV40er/Ras transgenes (**Figure 18**). At end stage, we collected terminal bleeds to isolate serum from tumor bearing animals for the purpose of assessing the single molecular diagnostic potential in a pre-clinical model. In addition to generating HIM tumors, this past year we also collected serum from mice bearing xenografts from ER+ breast cancers (MCF7 (N=10) and T47D (N=10) lines) ER-negative breast cancers (SUM1315, N=5). We also have collected weekly blood samples from mice engrafted with human breast cancer cells that can develop primary tumors and metastases (N=12).

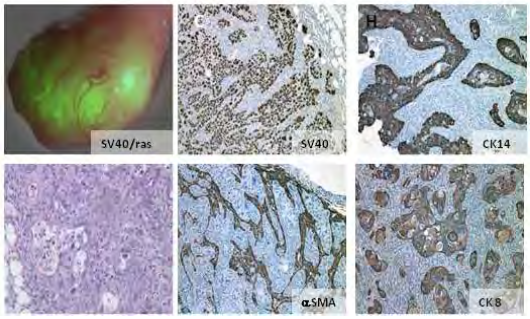


Figure 18: HIM Tumors. (A) GFP-whole mount, H&E and IHC analysis of tumors. At end stage, blood was collected from HIM tumor bearing animals. Magnification (H&E) 40x; (IF) 200x.

Since a low limit of detection (0.88 pg/mL, 22fM) and linear response using digital ELISA for measuring human EpCAM was successfully established, the assay was then applied to detecting EpCAM in the terminal bleeds obtained from HIM models with tumors derived from EpCAM-positive breast cancer cell lines MCF-7 and T47D. This experiment was done in order to assess if the assay can measure murine serum levels of EpCAM at the highest expected concentrations, before applying digital ELISA to measuring time-point serum samples for biomarker monitoring. Once the assay was applied to the terminal bleed samples, however, we found that we could only measure background levels and hence, EpCAM was undetectable in these samples (**Table 1**).

Sample Type	Sample Name	EpCAM Concentration (pg/mL)	Average % Active Microspheres (n=3)	%CV (n=3)
	Blank	0	0.16%	52%
Matrigel Control	Sample 627 (4x diluted)	Undetectable	0.08%	53%
T47D cell line	Sample 615 (4x diluted)	Undetectable	0.10%	55%
MCF7 cell line	Sample 606 (4x diluted)	Undetectable	0.07%	59%

Table 1: Digital ELISA values for HIM model serum samples measuring for EpCAM.

It is known that EpCAM is a marker that is produced by human epithelial cells and that a proteolytic enzyme called TACE (TNF-alpha converting enzyme) is required for the extracellular domain of EpCAM to be soluble[31]. It is possible that the HIM models may not produce this essential enzyme to allow EpCAM to circulate and be measured in serum. In order to investigate the presence of human TACE on the cell surface, levels of TACE in the tumor tissue should be measured using an ELISA or Western blot analysis. The findings from this experiment might indicate that the soluble form of EpCAM can be found in serum since the extracellular domain can be cleaved by the local presence of TACE.

Task 2. Apply single molecule diagnostic technique (developed in Task 1) to analysis of human serum samples (Years 3-5).

2a. Screen clinical samples obtained from Buchsbaum for presence of the markers that could be detected in HIM model (Months 25-30).

These studies have not been initiated per SOW.

2b. Iterate 2a-2d until a sufficient number of markers has been identified for further investigation. Some candidate markers are expected to either not be found in the clinical samples or will not be predictive of disease state. Other markers may be difficult to measure due to lack of suitable binding reagents. As these markers drop out, others will be added to ensure there is a sufficient number when sample set is expanded (2c) (Months 25-48).

These studies have not been initiated per SOW.

2c. Expand sample set and run clinical samples with all assays developed for all markers (Year 5).

These studies have not been initiated per SOW.

2d. Determine which markers in blood correlate with disease using data processing and computational methods available in the laboratory (Year 5).

These studies have not been initiated per SOW.

Task 3. Develop single cell analysis methods to determine composition of a primary tumor.

3a. Select approximately ten candidate markers (SNPs, proteins, etc.) of value for single breast cancer cell analysis in conjunction with collaborators (Months 1-9).

With new data just released in a recent publication in Nature,[32] we are now mining these data to select more informative SNPS for the Task.

3b. Detect presence of mtDNA by screening for many sequences using cultured cell lines and HIM tissue samples to select which markers to use. Will require development of mtDNA microarrays and sample screening (Months 6-18).

The selection of mtDNA markers is being performed in conjunction with the Sonenshein laboratory (see Sonenshein Task 1 below). The DNA assay developed above in Task 2 will be the basis for the assays we plan to use for Tasks 3b and 3c.

3c. Develop assays for the selected markers (Months 6-24).

The single cell studies described by the Chiu group below will facilitate our single cell analyses. While we are somewhat behind on Task 3, we expect to complete Tasks 3a, 3b, and 3c by the end of Year 2.

3d. Develop single cell assays for selected markers (Months 18-36).

These studies have not been initiated per SOW.

3e. Obtain tissue samples from HIM (Kuperwasser) and perform single cell analysis (Months 36-48).

These studies have not been initiated per SOW.

3f. Determine if one can observe rare cells with more aggressive genotypes or protein levels in HIM samples (Months 42-54).

These studies have not been initiated per SOW.

3g. Confirm presence of the single cells in human breast cancer biopsy samples obtained from Buchsbaum (Months 48-60).

These studies have not been initiated per SOW.

Daniel T. Chiu, PhD, *University of Washington, Seattle Campus Box 351700 Seattle, WA 98195-1700*

Task 1. Work with Walt lab to develop/refine single-molecule and single-cell techniques for analyzing protein and cell biomarkers (Months 1-36).

Task 1 *will use cultured cell lines, commercially available serum samples, and serum samples from HIM model.*

For Task 1, we have made good progress towards the detection and analysis of cell biomarkers, in particular, circulating tumor cells (CTCs). Using cultured cancer cells as a model system, we can now detect and recover with ~ 95% efficiency individual CTCs for analysis. CTCs have emerged as an important and valuable biomarker for the prognosis of breast cancer, and the sensitivity we have demonstrated makes CTCs a robust biomarker for prognosis and as an indicator of treatment efficacy. The high sensitivity has also the potential to make CTCs a diagnostic tool if they are present at sufficient levels in blood. Below, we describe each subtask in more detail.

***1a. Develop microfluidic devices for sample preparation and optical manipulation. Initial devices will contain only sample preparation module for use by Walt lab (Years 1-2*).**

We have developed a next generation microfluidic and optical device that can handle whole blood and then enrich for CTCs. This capability is useful both because CTCs are an important cell biomarker for breast cancer and also because CTCs may provide insight into protein biomarkers that are being evaluated in the Walt lab.

The schematic below shows the design and operation of the microfluidic device we developed. Error! Reference source not found.shows the overall layout of the chip. It contains several modules: Whole blood flows down the main channel and the presence or absence of CTCs within a ~ 2nL aliquot of whole blood is determined using sensitive fluorescence detection. If a CTC is present, the aliquot is sorted into a collection channel, where the CTC is further enriched and isolated from the ~ 10,000 blood cells present in the ~ 2nL aliquot. This additional enrichment and isolation is carried out using a series of microfabricated filters. The CTCs isolated from blood and trapped on the filter can be further analyzed.

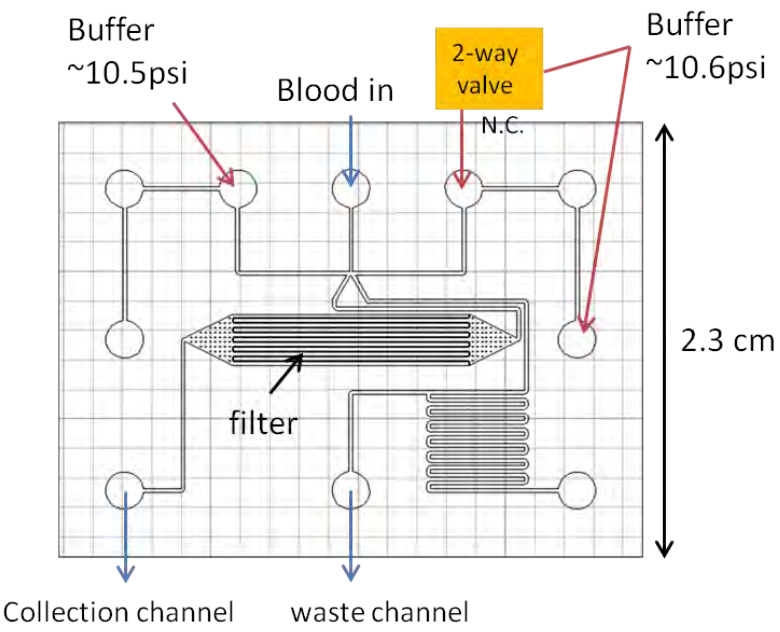


Figure 19: Schematic layout of the microfluidic chip for isolating cell biomarkers from whole blood of breast cancer patients. Whole blood flows down the main center channel, typically at a flow rate of ~ 50 ul/min. Together with the main blood flow, two side channels containing buffer also flow down the channels to surround the main blood flow. This is needed for sorting using flow displacement and for maintaining flow in the filtration region. The sorted cells of interest are further purified and isolated using a microfabricated filtration system.

One challenge we had to overcome in this development was fast and robust sorting of the identified blood aliquot that contained a CTC. To achieve the needed throughput, our sorting must be on the order of 1 ms. Additionally, to have the capacity and throughput to process many clinical samples in later years, our device must also be simple and rapid to fabricate. These considerations prompted us to develop a sorting scheme that employs off-chip valves, because on-chip valves are typically tedious and expensive to make and often not sufficiently robust. We have evaluated numerous options and identified a working solution to overcome this issue. **Figure 20** shows one sorting scheme that we developed, which does not involve any modifications to our microfluidic design, maintains a one-layer chip format and is easy to fabricate because it involves only one replication and bonding step. To test the robustness of this sorting scheme, we placed two line-confocal detection regions, one immediately before the sorting junction and one immediately after. We found that we have 100% sorting efficiency based on thousands of sorting events. This sorting is also useful as an active element for other applications that involve sample preparation of whole blood from patients.

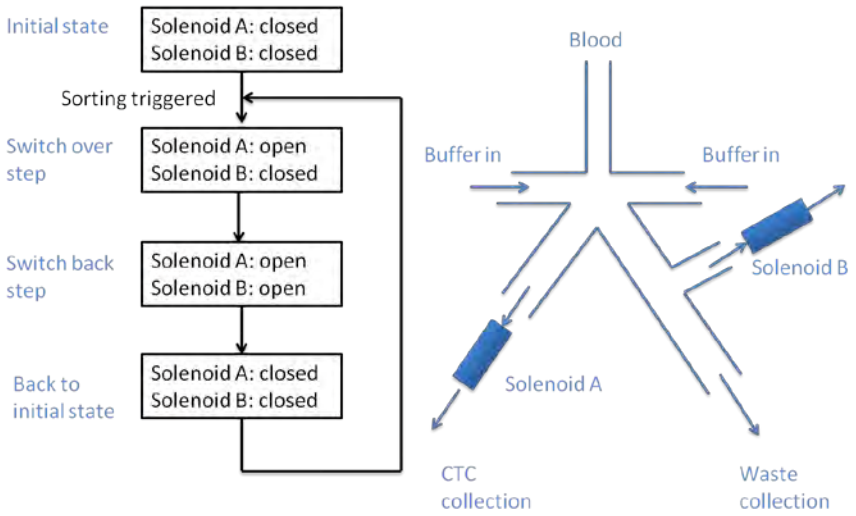


Figure 20: Schematic showing the sorting scheme we developed for the active processing of whole blood, in which aliquots of whole blood that contain CTCs are sorted into a sample collection reservoir for downstream processing. The left shows the valving sequence and the right shows the simplified layout of the various elements.

Finally, after the blood aliquots containing CTCs are sorted, we use an on-chip microfabricated filtration system to further purify the CTCs from blood cells. This is necessary because each sorted aliquot that contains a CTC will also contain ~ 10,000 blood cells. The filtration system effectively removes these blood cells and leaves the CTCs retained on the filter. **Figure 21** shows a bright-field (left panel) and fluorescence (right panel) image of the filter with trapped cancer cells. Once retained on the filter, a number of downstream analysis and processing protocols of the CTCs are possible.

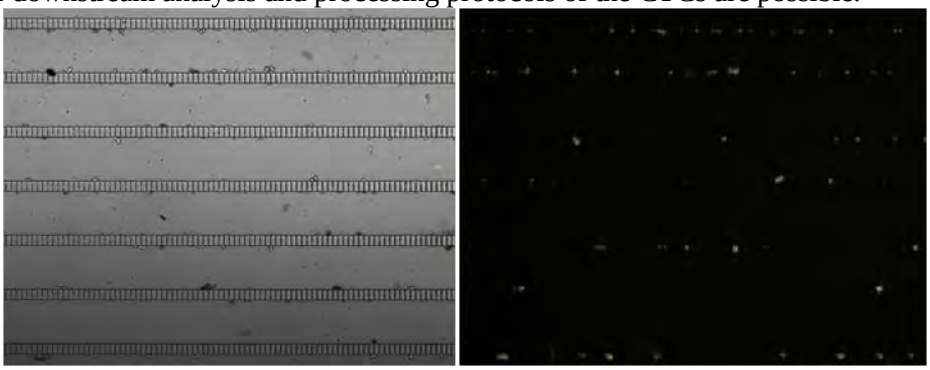


Figure 21: Image of retained cancer cells on the microfabricated filtration system. Left panel is a bright field image and the right panel is the corresponding fluorescence image.

In summary, in this subtask 1a, where we devoted most of our efforts in the past year, we have completed a microfluidic device for the sample preparation of whole blood, where we are able to detect and isolate a single cultured cancer cell spiked into 1mL of whole blood containing ~ 5 billion blood cells with a ~ 95% recovery efficiency. Furthermore, using whole blood from healthy donors, we determined we have a zero false positive rate; that is, we have not detected a single false positive event out of the dozen samples we ran. The technology we developed here is not only useful for the isolation of CTCs from blood, but also for other sample preparation needs where whole blood is involved.

1b. Integrate assays and optical methods developed in Walt lab into a microfluidic device for the analysis and profiling of biomarkers (Years 1-2).

The first step and the simplest approach to integrate the single-molecule array technology developed in the Walt lab for the analysis of CTCs is to remove the CTCs from the sample preparation chip described above so that CTCs can be lysed and the important but low abundant protein biomarkers

derived from the CTCs can be detected and quantified using the single-molecule array technology. Towards this goal, we have developed methods for the isolation of individual CTCs from the sample preparation chip. **Figure 22** below shows our capability, where we use a micropipette to remove individual selected cancer cells from the chip, after which we are able to seed the single cancer cell on a photo-etched coverslip and then culture the removed single cancer cell through multiple rounds of cell division. With this capability, we will be able to place single CTCs into a small volume, lyse the cell, then analyze the protein content using the single-molecule array technology. The use of a micropipette does constrain the throughput of this method, but given the rarity of CTCs in blood (a few per mL of whole blood), throughput is normally not an issue.

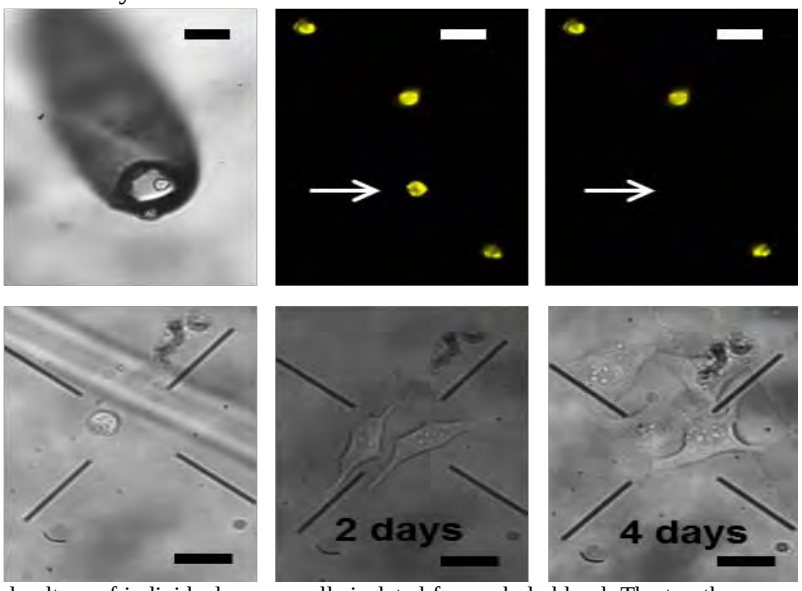


Figure 22: Removal and culture of individual cancer cells isolated from whole blood. The top three panels show the process of using a micropipette to remove a single cancer cell identified in the fluorescence image. The bottom three panels show the seeding of the removed cell onto a photo-etched coverslip and subsequent culture and clonal expansion of the single cancer cell.

1c. Develop integrated microfluidic and optical techniques for single cell analysis using model cell lines (Years 1-3).

For this task, we have several different approaches, such as digital PCR and digital protein (subtask 1b) analysis of individual CTCs. However, our main focus in the past year has been the development of an immuno-histochemistry approach to image different types of proteins present in individual CTCs. **Figure 23** shows the approach, in which the CTCs retained in the sample preparation chip (subtask 1a) is first imaged in bright field or Normaski (top panel), after which we label the cells with fluorescent antibodies. Here, the microfluidic device offers easy perfusion of the antibody solution over the cells because the cells are already retained on the filter. Because of the small volume of the filtration area, we are also able to use very small amounts of antibody solution, which is practically useful because of the expensive nature of antibodies. But more importantly, because of the small filtration area, we are also able to image one set of fluorescent antibodies, bleach the sample, then re-label with another set of antibodies and repeat the imaging. In this way, we are able to image a large set of different types of proteins and to dissect the key proteins that malfunction in these cancer cells. With our optical instrument, we are able to image five colors, and thus each repetition of labeling and imaging allows us to image five types of proteins. Again, the combination of easy perfusion and cell labeling together with the small filtration area that can be covered with one or a few field-of-view of the microscope enables us to image tens of different types of proteins with excellent throughput.

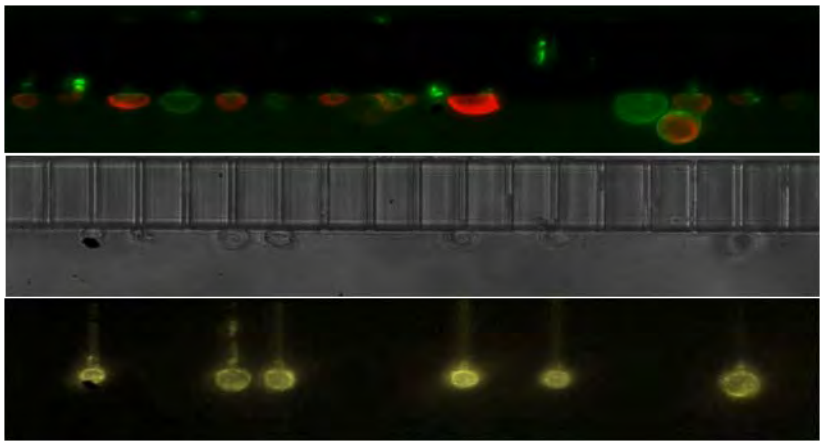


Figure 23: Images of cancer cells retained by the microfabricated filter. The middle panel shows a bright-field image, the bottom panel shows a fluorescence image in the yellow/orange channel, while the top panel shows an example fluorescence image in the green and red channel.

Task 2. Work with the Walt lab to apply these methods to breast-cancer patient samples for the detection and validation of protein and cell biomarkers *(Months 25-60)*.

2a. Develop/submit amended proposal to University of Washington IRB to permit secondary use of currently archived patient samples (Months 1-12).

We have worked with the University of Washington's IRB and with HRPO and we have now obtained permission from both to use our over 100 archived breast cancer patient samples for this project. This task is now complete. For record, pasted below in italic is the e-mail we received from HRPO indicating we can use the archived samples for this project because they are not subject to 45 CFR 46.

Date: Thu, 26 Jul 2012 07:46:59 -0400
Subject: A-16787.1, CORRECTED HRPO Determination Memorandum (Proposal Log Number BC100510, Award Number W81XWH-11-1-0814) (UNCLASSIFIED)
SUBJECT: CORRECTED Determination of Not Research Involving Human Subjects for the Protocol, "Single-Cell Technologies for Detection and Eradicating Circulating Tumor Cells and DNA," Submitted by Daniel T. Chiu, PhD, University of Washington, Seattle, Washington, on Behalf of the Proposal, "Development of Technologies for Early Detection and Stratification of Breast Cancer," Submitted by David R. Walt, PhD, Tufts University, Medford, Massachusetts, Proposal Log Number BC100510, Award Number W81XWH-11-1-0814, HRPO Log Number A-16787.1

1. The subject project, supporting documents, and clarifications received on June 2012 in the US Army Medical Research and Materiel Command, Office of Research Protections, Human Research Protection Office (HRPO) have been reviewed for applicability of human subjects protection regulations.

2. The project involves analysis of banked de-identified serum samples. The dataset does not contain any identifiers or any set of variables that might allow for the identification of individuals. The investigators in this Multi-Team Award cannot readily ascertain the identity of the individuals linked to the coded private information or specimens because there is an agreement prohibiting the release of the key to the investigators.

3. The University of Washington Human Subjects Division or Subcommittee has determined that this project does not meet the criteria for human subjects research. This determination indicates that the project is not subject to 45 CFR 46 and does not require further review by the IRB.

4. The HRPO has determined in accordance with 32 CFR 219.102(f). The project may proceed with no further requirement for review by the HRPO. The HRPO file for this specific project will be closed.

5. In the event there is a change to the subject project or statement of work (SOW), the Principal Investigator must notify the Grants Officer's Representative (GOR) and send a description of the change to the HRPO at hrpo@amedd.army.mil referencing both the proposal log number and the HRPO log number listed in the "Subject" line above. The HRPO will re-open the protocol file if necessary.

Any changes to the SOW that the GOR determines could involve research activities must be reviewed by the HRPO prior to approval by the Contracting Officer/Grants Officer.

6. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer/Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting officer regarding the expenditure of funds for your project.

7. Further information regarding this review may be obtained by contacting Diana Weld, MS, Human Subjects Protection Scientist, at 301-619-6247 or diana.weld@us.army.mil.

SARAH L. DONAHUE, PhD, MPH, CIP

Human Subjects Protection Scientist
Human Research Protection Office
Office of Research Protections
US Army Medical Research and Materiel Command

2b. Apply sensitive techniques (1a and 1b) for the retrospective analysis and validation of biomarkers from archived patient samples (Months 25-60).

We propose to use archived blood plasma samples from breast cancer patients. One hundred archived blood plasma samples already exist in the Chiu lab from a previous three-year study on detection of biomarkers in breast cancer patients.

2c. Apply sensitive techniques (1a and 1b) for the prospective analysis and validation of biomarkers from patient samples (Months 36-60).

We propose to enroll a cohort of about 25 breast cancer patients where each patient will have on average two blood draws per year, for a total of 100 blood draws. The Chiu group has a current approved IRB protocol for prospective collection of serum from human subjects with breast cancer, previously funded (through Dec 31, 2010) by the Life Science Discovery Fund (LSDF). The current IRB protocol is valid until July 2012, and will be updated based on DOD funding once the project has started.

2d. Apply techniques developed for the prospective analysis of cancer cells (1a and 1c) from patient samples obtained in 2c with single-cell resolution (Months 36-60).

These studies have not been initiated per SOW.

Charlotte Kuperwasser, PhD, *Tufts University School of Medicine, 750 Washington St., Box 5609 Boston, MA 02111*

Task 1. Early Detection (Months 1 to 36)

1a. Utilize the 'Human-In-Mouse' breast tumor model to determine if one can identify biomarkers of early tumor progression and tumor growth. Collect blood and breast tissues at various stages of progression: Normal, hyperplasia, DCIS, invasive cancer (Months 1-18). HIM tumors will be created from human breast epithelial cells collected from discarded tissues of women who have undergone reduction mammoplasty surgeries. For more information/details on model: Nature Protocols, 2006, 1, 595-599.

As described above, we generated orthotopic humanized tissue-transgenic breast cancers using mammary epithelial cells obtained from reduction mammoplasty tissues from patients undergoing elective surgery. We focused on generating HIM tumors using SV40er/Ras transgenes as this approach models tumor progression in a well-defined manner. This past year, we focused on obtaining samples from last stage invasive cancers (N=10). At end stage, we collected terminal bleeds to isolate serum from tumor bearing animals for the purpose of assessing the single molecular diagnostic potential in a pre-clinical model. We have provided these samples to the Walt Laboratory for analysis. Moving forward, we will concentrate on collecting blood from earlier stages including DCIS and hyperplasia. In addition, as we revise the biomarker list, we will also create HIM tumors harboring the specific gene mutations (eg. MAP3K, ErbB2).

1b. Utilize the 'Human-In-Mouse' breast tumor model and inject different cancer to normal cell ratios—1:10, 1:100, 1:100 (Months 18-32)

Predicted Total mice =(80x2replicates)=160; Predicted total number of reduction mammoplasty tissues= 10

These studies have not yet been initiated per SOW.

Task 2. Response to chemotherapy and hormone therapy (Months 24-48).

2a. Inject traditional breast cancer cell-line based xenografts into mice using hormone receptor positive lines and hormone receptor negative and Her2+ lines (MCF7, T47D, HCC1418, SUM225, BT20, SUM149, SUM159)(Months 24-36). Human breast cancer cell lines are all commercially available and previously characterized. For more information/details on these cell line-based xenografts see: Breast Cancer Research, 2010;12(5):1-17.

Cell-line based xenografts have been initiated and terminal blood collections have been made with MCF7, T47D and SUM1315 lines (N=10, 10, 12, respectively). Chemotherapy and hormone therapy treatments and studies have not yet been initiated.

2b. Allow tumors to form and reach 5mm and assess serum levels of EMT/CSC markers. (Months 24-36)

These studies have not yet been initiated per SOW.

2c. Treat animals with chemotherapy (Paclitaxel/Taxol) or anti-estrogen (Tamoxifen) and measure serum levels of EMT/CSC markers and mtDNAs during tumor regression and after cessation of therapy.

These studies have not yet been initiated per SOW.

2d. Continue to monitor tumor growth. (Months 36-48)

These studies have not yet been initiated per SOW.

2e. Determine whether the levels of EMT/CSC markers correlate to response to therapy or to tumor recurrence (Months 36-48).

Predicted Total mice =(30x2replicates)=60

These studies have not yet been initiated per SOW.

Task 3. Recurrence prediction (Months 36-60)

These studies have not yet been initiated per SOW.

3a. Create tumors using the 'Human-In-Mouse' breast tumor model. Allow tumors to form and reach 5-8mm in diameter and assess serum levels of oncogenes used to create tumors (Months 36-50).

These studies have not yet been initiated per SOW.

3b. Surgically resect the tumors and measure blood serum levels of EMT/CSC markers as well as mitochondrial DNAs during tumor regression and after cessation of therapy after surgery (Months 36-50).

These studies have not yet been initiated per SOW.

3c. Monitor mice and measure the levels of EMT/CSC markers weekly to determine if they increase or correlate with regrowth of tumor (Months 48-60).

Predicted Total mice=(80x2replicates)=160;Predicted total number of reduction mammoplasty tissues= 10

These studies have not yet been initiated per SOW.

Gail Sonenshein, PhD, *Tufts University School of Medicine 150 Harrison Avenue Boston, MA 02111*

In Year 1, the Sonenshein group proposed the following experiments in Task 1 of the SOW:

Task 1. Perform mitochondrial DNA mutational analysis.

1a. Isolate mitochondria from cultured mouse and human mammary breast epithelial or cancer cells that exist in our laboratory and extract DNA and subject it to massively parallel sequencing and bioinformatics to identify mutations. (Months 1-8).

1b. Elucidate the nature of mtDNA mutants in pre-existing mammary tissue of MMTV-c-Rel transgenic mice transformed by carcinogen treatment (prepared by our laboratory). Isolate mitochondria from normal tissue and from tumors, extract DNA and subject it to massively parallel sequencing (Months 8-12).

1c. Compare mtDNA from normal mouse mammary tissue and from tumor tissue using bioinformatics to identify mutations. (Months 10-16).

In addition, we proposed to test the hypothesis that mtDNA can be used to test for breast cancer regression and then recurrence (Task 3). We are well along the way on Task 1 and have laid the groundwork for our component of Task 3 (specifically Task 3b).

To begin the project, we needed to identify primers that are specific for mitochondrial DNA and that are unable to amplify the pseudogenes that exist in the nucleus. After consultation with a number of groups, we chose to use the mtDNA specific primers developed by the laboratories of Taylor and Turnbull, which allow for amplification of mtDNA from a single cell without amplification of the nuclear pseudogenes (R.W. Taylor, G. Taylor, S. Durham, and D.M. Turnbull. Nucleic acids research, 2001, 29,

e74-4). As a test of their ability to prime nuclear pseudogene DNA, we obtained rho zero cells from Eric Schon (Columbia University). The rho zero cells have almost no mitochondrial DNA but retain the nuclear pseudogenes, and thus serve as an excellent negative control. We also received matching control 143B cells. DNA amplification with various primer sets was profoundly reduced with the rho zero cell DNA, as reported by Taylor et al. (2001), with an occasional non-specific band seen. In contrast, effective amplification of the control DNA was readily detected (**Figure 24**). Thus, we propose to use the mitochondrial primers of Taylor and Turnbull.

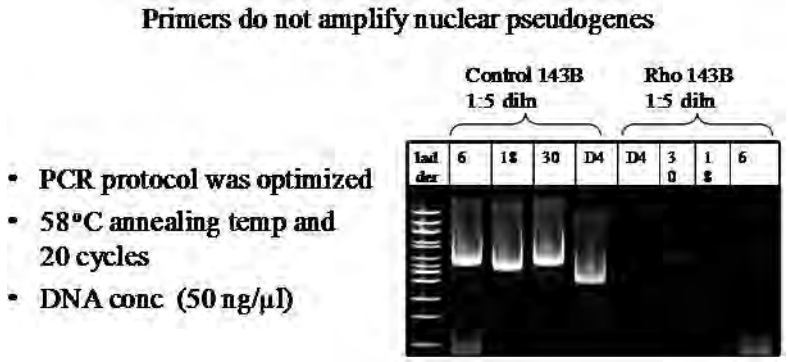


Figure 24: Mitochondrial specific primers of Taylor and Turnbull amplify mitochondrial DNA but not nuclear pseudogenes.

1a. Isolate mitochondria from cultured mouse and human mammary breast epithelial or cancer cells that exist in our laboratory and extract DNA and subject it to massively parallel sequencing and bioinformatics to identify mutations. (Months 1-8).

1c. Compare mtDNA from normal mouse mammary tissue and from tumor tissue using bioinformatics to identify mutations. (Months 10-16).
These studies will elucidate the alterations in the mitochondrial DNA induced by treatment with the carcinogen DMBA, and allow us to compare its effects in vitro with those in whole animals.

We next performed the analysis described in Task 1a. Specifically, we isolated mitochondrial DNA from a panel of untransformed breast epithelial and breast cancer cell lines in the laboratory, identified in **Table 2**. These included Estrogen receptor (ER)α positive and ERα negative, and two inflammatory breast cancer (IBC) lines. In addition, to begin to identify mutations associated with carcinogen treatment, we analyzed benzo[a]pyrene (BP1) and DMBA (D3) carcinogen transformed derivatives of MCF10A. All of the mtDNA samples were subjected to DNA sequencing and the specific sequence of 16,569 bp mtDNA determined.

Untransformed	ERα+	ERα-	Inflammatory breast cancer (IBC)	Carcinogen treated
HMEC	MCF7	BT549	SUM149	BP1
MCF10A	T47D	MDA-MB-231	SUM190	D3
		Hs578T		

Table 2: Table describing the panel of untransformed breast epithelial and breast cancer cell lines from which mitochondrial DNA was isolated.

We compared the sequencing with mutations that are seen in human breast cancer specimens. We observed **39** mutations that are common with breast cancer patient samples (**Table 3**). A linearized "view" of the mtDNA is shown in **Figure 25**. Interestingly the two IBC cell lines each have a unique mutation in a tRNA gene that was not observed in any other kind of breast cancer cell line. These mutations will be used in diagnosis of blood from mice carrying tumors derived from the cell lines in Task 3 below.

73 A>G	
152 T>C	**7028 C>T**
185 G>A	7521 G>A
189 A>G	8701 A>G
195 T>C	**8860 A>G**
198 C>T	9540 T>C
228 G>A	10873 T>C
263 A>G	10398 A>G
295 C>T	11251 A>G
311 INSERT C	11719 G>A
489 T>C	12612 A>G
523 A-DEL	12705 C>T
750 A>G	13650 C>T
769 G>A	16278 C>T
1438 A>G	16294 C>T
2706 A>G	16311 T>C
3010 G>A	16319 G>A
4216 T>C	**16519 T>C**
4769 A>G	**15326 A>G**
3594 C>T	13263 G>A

Table 3: MtDNA mutations found in breast cancer cell lines that are common with those in patient tumors. **Bold** font indicates mutations found in both ER positive and negative cell lines.

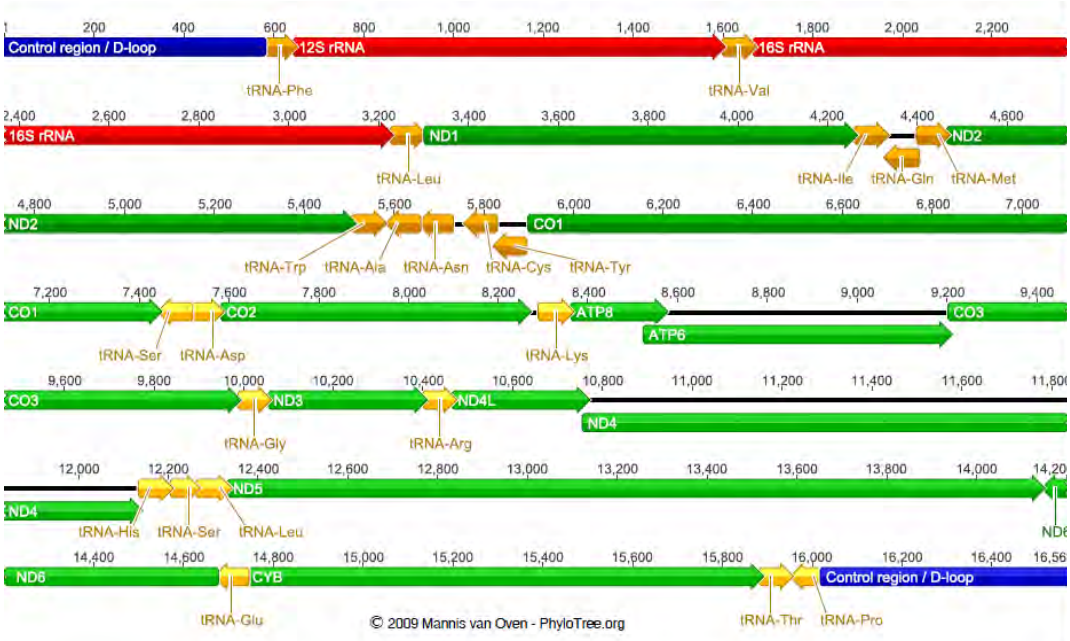


Figure 25: Linearized view of the mtDNA with positions of the 13 genes, 22 tRNAs, 2 rRNAs and the control D-loop indicated.

We also studied the effect of hypoxia (1% oxygen) on mtDNA mutations as it has been reported to cause mutations in the nuclear genes. We found that even after prolonged exposure to Hypoxia, there were no additional mtDNA mutations observed. Recently another lab has published a similar finding.[33]

1b. Elucidate the nature of mtDNA mutants in pre-existing mammary tissue of MMTV-c-Rel transgenic mice transformed by carcinogen treatment (prepared by our laboratory). Isolate mitochondria from normal tissue and from tumors, extract DNA and subject it to massively parallel sequencing (Months 8-12).

In order to be able to analyze DNA derived from human cells in mice, described in Tasks 2 and 3, we designed mtDNA primers specific for human DNA that do not recognize mouse DNA. We confirmed the specificity of these primers using DNA from human cells (D3 carcinogen transformed human MCF10A cells) vs mouse cells (MMTV-c-Rel tumor-derived cell line) (**Figure 26**). Thus, we will be able to use these primers to look for characteristic mtDNA mutations in the tumors in mice derived from human breast cancer cells, and avoid potential pitfalls due to the presence of mouse DNAs.

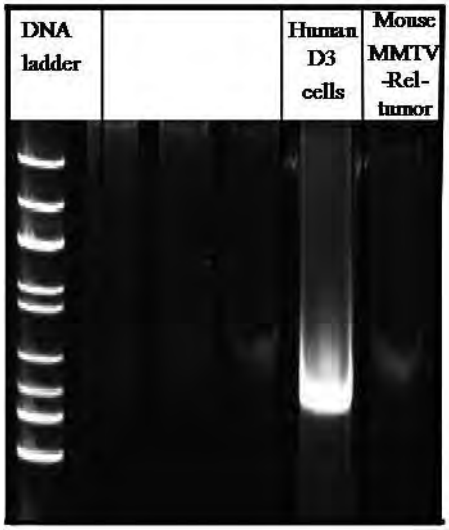


Figure 26: Specific primers can recognize human and not mitochondrial DNA.

Task 2. Characterize mtDNA mutations resulting from oncogene expression in murine breast tumors (Months 16-24).

2a. Compare mt DNA from mammary tissue vs. tumors or derived cell lines of transgenic mice driven by oncogenes implicated in breast cancer using 8 matched pair samples for each oncogene. Samples obtained from Kuperwasser (Months 16-24).

These studies have not yet been initiated per SOW.

2b. Characterize mtDNA alterations in tumor lines that will be utilized by the Kuperwasser laboratory in their 'HIM' analysis. DNA samples will be analyzed as in Task 1 above (Months 18-24).

Data from this analysis will provide information on the effects of oncogene activation on mtDNA integrity and provide the baseline analysis for the recurrence studies outlined below in Tasks 3 and 4.

Task 3. Recurrence prediction: Determine whether mtDNA mutations can be used to test for breast tumor regression in mice (in collaboration with Kuperwasser) (Months 36-60).

These studies have not yet been initiated per SOW.

3a. mt DNA from tumor samples provided by the Kuperwasser laboratory using the HIM breast tumor model. Tumors will be initially removed after they have been allowed to reach 5-8 mm in diameter and then at the end of the experiment (Months 18-30).

These studies have not yet been initiated per SOW.

3b. DNA will be isolated from serum collected before resection and weekly after resection that will be provided by the Kuperwasser laboratory (Months 18-30).

As a pre-requisite for analysis of regression and recurrence, we need to be able to amplify mtDNA from whole blood of mice bearing a human tumor. To develop this technique, blood from mice containing MDA-MB-231 tumors of ~1 cm^3 was analyzed. We isolated total DNA from the blood and subjected it to the PCR protocol described above. Unfortunately, we found that the analysis showed multiple additional bands. Thus we sought to improve the fidelity of the PCR process and tested several high fidelity polymerases. We observed that the New England Biolabs (NEB) Q5 High Fidelity polymerase successfully amplified the DNA and gave reproducible, specific bands as seen with the cell DNA. Thus, we will use the NEB Q5 polymerase with DNA extracted from blood. We are in the process of testing the sensitivity of the isolation and amplification procedures using blood from mice with MDA-MB-231 cell-derived tumors that are only beginning to be palpable to verify our ability to isolate and detect mtDNA when tumor burden is low.

3c. In conjunction with the Walt lab, identify tumor-derived mtDNA obtained from Kuperwasser in the serum and determine whether the levels correlate with tumor regression and recurrence (Months 24-40).

These studies have not yet been initiated per SOW.

Task 4. Response to chemotherapy and hormone therapy (in collaboration with Kuperwasser) (Months 30-60).

4a. mtDNA will be isolated from tumor and blood samples provided by the Kuperwasser laboratory (Task 3) (Months 24-48).

These studies have not yet been initiated per SOW.

4b. Analyze the mtDNA for the appearance of hallmark mutations using assays developed in Walt lab to allow us to monitor the effects of initiation and cessation of therapy on mtDNA in the tumors and serum and determine whether the levels correlate with tumor regression and recurrence (Months 30-60). Findings will be applied to the analysis of patient samples in collaboration with Buchsbaum.

These studies have not yet been initiated per SOW.

New Task.

We have also worked on ADAM8, which was not part of the original SOW but may be a valuable biomarker for the Walt Lab to develop an assay.

As discussed above, miRNA represent potential biomarkers for breast cancer detection as they are released from tumor cells and can be present stably in the blood stream. We have identified ADAM8 (ADAM metallopeptidase domain protein 8) as a key downstream mediator of a RelB NF-κB pathway that promotes a more aggressive phenotype of breast cancer. ADAM8 belongs to a disintegrin and metalloprotease (ADAM) protein family, which includes membrane-anchored proteins mediating cell adhesion, migration, signaling and proteolysis of extracellular matrix. To date, we have shown that *ADAM8* is overexpressed in breast tumor samples *vs* normal breast tissue, especially in ER-negative and triple negative breast cancers. Interestingly, *ADAM8* levels correlate with metastatic relapse in patients. Using immunohistochemistry, our collaborators in France have shown that 34% of triple negative tumors and 48.2% of breast metastases are positive for ADAM8 staining. *In vitro*, ADAM8 plays an essential role in migration and invasion properties, and promotes invasive outgrowth and anchorage-independent growth in 3D-assays. *In vivo*, ADAM8 knockdown strikingly prevented tumor development and spreading of circulating tumor cells from MDA-MB-231 cells in an orthotopic mammary mouse model. Thus, we initiated a project to identify miRNAs regulated by ADAM8. To this end, we knocked down *ADAM8* RNA levels in MDA-MB-231 cells using either of two ADAM8 siRNAs for 72 hours. RNA was

isolated from two independent experiments (runs) and subjected to TaqMan low density miRNA array cards that can detect all known miRNAs. We observed significant decreases in 36 miRNAs (**Table 4**). We selected several of these miRNAs and confirmed the results using Real-time Q-PCR. We are in the process of measuring the (1) levels of these miRNAs in breast cancer cells with high vs low ADAM8 and following knockdown or ectopic ADAM8 expression; (2) levels of expression of these miRNA in tumors derived from control shRNA or shADAM8 MDA-MB-231 cells and circulating in the blood of these mice. These experiments will identify miRNA markers of ADAM8 overexpression that can be used for early detection of metastasis.

	miRNA ID	fold change in run 1	ct values control	ct values sample	fold change in run 2	ct values control	ct values sample
1	**hsa-let-7f-000382**	**-5.35**	32.926	33.742	**-2.93**	34.6	36.5
2	**hsa-let-7g-002282**	**-3.33**	29.97	31.19	**-2.06**	28.526	29.04
3	**hsa-miR-16-000391**	**-2.86**	23.342	24.061	**-2.44**	24.44	25.43
4	**hsa-miR-18b-002217**	**-5.32**	38.111	undetermined	**-3.95**	38.54	undetermined
5	**hsa-miR-19a-000395**	**-5.08**	29.08	30.9	**-2.16**	28.597	29.062
6	**hsa-miR-24-000402**	**-2.87**	24.44	25.43	**-2.1**	22.918	23.4
7	**hsa-miR-26b-000407**	**-4.83**	32.93	34.67	**-3.86**	30.99	32.357
8	**hsa-miR-29c-000587**	**-2.43**	28.9	29.66	**-2.14**	27.43	27.97
9	**hsa-miR-95-000433**	**-2.22**	36.35	36.98	**-5.75**	33.522	35.452
10	**hsa-miR-98-000577**	**-2.28**	33.06	33.73	**-2.58**	31.76	32.374
11	**hsa-miR-106b-000442**	**-4.52**	29.99	31.65	**-2.4**	28.93	29.67
12	**hsa-miR-125a-5p-002198**	**-3.94**	25.68	27.13	**-2.96**	24.65	25.75
13	**hsa-miR-125b-000449**	**-2.92**	30.74	31.76	**-2.26**	28.99	29.6
14	**hsa-miR-146a-000468**	**-3.01**	26.95	28.018	**-3.46**	25.97	27.17
15	**hsa-miR-152-000475**	**-8**	37.53	undetermined	**-3.77**	32.744	34.097
16	**hsa-miR-191-002299**	**-3.13**	23.92	25.04	**-4.08**	24.65	23.193
17	**hsa-miR-192-000491**	**-7.81**	31.71	34.15	**-2.85**	30.65	31.6
18	**hsa-miR-193a-5p-002281**	**-17.86**	36.03	39.65	**-4.07**	35.4	37
19	**hsa-miR-195-000494**	**-3.3**	33.28	34.48	**-2.65**	31.25	32.05
20	**hsa-miR-222-002276**	**-2.73**	23.32	24.25	**-2.15**	22.38	22.8
21	**hsa-miR-324-3p-002161**	**-3.83**	31.9	33.31	**-2.75**	31.05	31.94
22	**hsa-miR-330-000544**	**-18.18**	35.37	39.03	**-10.53**	34.09	37.07
23	**hsa-miR-335-000546**	**-3.51**	35.08	36.4	**-2.12**	33.64	34.16
24	**hsa-miR-362-001273**	**-2.72**	33.46	34.38	**-4.12**	33.65	35.15

25	**hsa-miR-374-000563**	**-2.51**	28.16	28.97	**-2.6**	27.6	28.4
26	**hsa-miR-449-001030**	**-28.57**	34.96	undetermined	**-47.62**	35.72	undetermined
27	**hsa-miR-452-002329**	**-5.21**	32.99	34.91	**-5.43**	31.33	33.15
28	**hsa-miR-454-002323**	**-2.18**	26.52	27.13	**-2.53**	25.79	26.58
29	**hsa-miR-532-001518**	**-7.52**	34.69	37.077	**-5.1**	32.69	34.48
30	**hsa-miR-574-3p-002349**	**-2.08**	26.26	26.79	**-2.04**	25.29	25.78
31	**hsa-miR-744-002324**	**-3.48**	32.55	33.83	**-2.65**	30.54	31.4
32	**hsa-miR-30a-3p-000416**	**-4.5**	19.89	25.6	**-10**	24.15	25.95
33	**has-24-2#-002441**	**-8.33**	28.94	35.54	**-14.08**	33.1	35.3
34	**has-320b-002844**		30.688	undetermined	**-142**	24.92	35.65
35	**has-93#-002134**	**1**	24.37	28.02	**-25.64**	26.7	29.83
36	**hsa-20a#-002437**	**-3.04**	29.61	34.76	**-31.25**	33.926	37.376

Table 4: Effects of ADAM8 knockdown on miRNA levels in MDA-MB-231 cells

Rachel Buchsbaum, MD, *Tufts Medical Center, 800 Washington St, Box 245 Boston, MA 02111*

Task 1. Determine effect of tumor microenvironment on serum markers of metastasis *in the HIM model* (Months 1-36).

These studies have not yet been initiated per SOW.

1a. Develop mammary fibroblast lines with range of Tiam1 (both down- and up-regulated). (Months 1-6)

This Task has been completed. We have generated mammary fibroblast lines with stable silencing of Tiam1 expression, stable over-expression of Tiam1, and corresponding control lines. We have also used these lines in our 3D co-culture model to verify the effects on co-cultured mammary tumor cell lines in terms of migration, invasion, and modulation of cancer stem cell-like populations.

1b. Utilize HIM breast tumor model in collaboration with Dr. Kuperwasser. Allow tumors to reach 10mm in diameter. Monitor mice weekly and assess serum levels of biomarkers of early tumor progression and tumor growth using single molecule diagnostic assays developed in Walt lab (see Walt task 1; Months 6-18). Numbers of mice needed = 10 different experimental conditions x 20 mice per experimental cohort = 200 mice

We have established tumor cell implantation in the HIM model in 64 mice to date and are currently monitoring for tumor development.

1c. Surgically resect tumors and perform in vivo bioluminescence imaging to monitor development of tumor metastasis weekly (Months 18-36).

These studies have not yet been initiated per SOW.

1d. Collect weekly serum samples and measure serum levels of biomarkers in collaboration with Walt lab to determine if there is correlation with development of metastatic disease. (Months 6-36)

Serum collections should begin within the next 2 weeks.

Task 2. Perform retrospective clinical trial to validate candidate markers from Walt single molecule studies using banked repository samples. (*Months 1-60*)

This task is largely on hold until candidate markers have been identified and validated using the Walt single molecule studies, as successful application to access banked samples from current repositories will require supporting pre-clinical data. Similarly, determining sample size will be most accurately done once the sensitivity and specificity of the assay for the specific candidate marker(s) has been determined.

2a. Work with statisticians to determine needed sample size for retrospective trial (Months 1-3).

2b. Identify repository study populations with available banked serum samples and adequate duration of follow-up to permit retrospective studies (e.g. Nurses Health Study, NCI repositories, or commercial sources such as Bioserve Human Serum Repository) (Months 3-9).

2c. Develop/submit research proposals and regulatory documents to obtain sample access from repositories (Months 10-16).

2d. Develop/submit proposal to Tufts Medical Center IRB for retrospective study of de-identified repository samples described in 2c; submit regulatory documentation to DOD (Months 17-24).

These studies have not yet been initiated per SOW.

2e. Obtain samples from repositories (described in 2b, 2c, 2d) for analysis of serum markers identified through the HIM model studies, and provide to Walt laboratory (see Walt Task 2a-d; Months 25-60).

These studies have not yet been initiated per SOW.

2f. Analyze results of candidate marker screening of banked samples with regard to correlation with clinical outcomes (Months 30-60).

These studies have not yet been initiated per SOW.

Task 3. Perform prospective clinical trial for predictive and prognostic markers in women with newly diagnosed breast cancer (Months 1-60).

3a. Work with statisticians to determine estimate of sample size for prospective trial. (Months 1-6)

We have determined that these samples will be collected prospectively under the aegis of the Tufts Medical Center Cancer Center Tissue Repository. The goal is to bank serum samples from all women with newly diagnosed breast cancer, as well as from women with active metastatic breast cancer. This is being coordinated with the multi-disciplinary services involved in the care of breast cancer patients, including the surgical oncologists, medical oncologists, and pathologists. The Tissue Repository has an active and approved IRB protocol (PI: Andreas Klein). This protocol has been revised to include serial sample collection as specified below, as well as to include residents participating in the care of the patients to obtain consent for serum banking (revised protocol attached here). The corresponding consent form for the serum banking is currently being revised.

3b. Develop and submit proposal to IRB for prospective study of newly collected serum samples for tumor markers from women diagnosed at Tufts Medical Center with newly diagnosed breast cancer; submit regulatory documentation to DOD (Months 1-12).

3c. Coordinate with Tufts Medical Center affiliate institutions to open multi-center trial to facilitate enrollment (Months 1-12).

3d. Enroll patients and gather serum samples: at diagnosis, post-treatment, and every 6 months after treatment for 5 years (Months 12-60 and then ongoing).

3e. Submit amended proposal to IRB regarding accurate sample size. The final number of human subject samples will be determined based on the sensitivity of the assay we develop and the number of markers to be tested, once validated in the HIM model (see Walt Task 1a-c; Months 18-30).

3f. Monitor for breast cancer recurrence and metastasis and correlate with levels of serum tumor markers (Months 12-60 and then ongoing).

3g. Collaborate with statisticians on data analysis at interim time points (Months 12-60 and then ongoing).

Task 4. Obtain clinical tumor samples for single cell analysis to determine tumor composition (see Walt task 3) and analysis of mtDNA (see Sonenshein task 4). (Months 1-60)

These studies have not yet been initiated per SOW.

4a. Work with breast surgeons and pathologists at Tufts Medical Center to develop technique for harvesting cells in fluid phase from fine needle aspirate (FNA) samples from murine tumors in HIM model (Months 1-12).

4b. Develop and submit proposal to IRB for collection and prospective study of newly collected breast cells obtained from human subjects via FNA as in task 4a; submit regulatory documentation to DOD (months 12-24).

4c. Recruit human subjects, obtain samples, and provide to Walt lab for single cell analysis studies (Months 25-36).
This study will initially be proposed as a pilot study of samples from 20 non-cancer and 20 breast cancer subjects, numbers that can practicably be obtained within a 12 month period.

4d. Work with statisticians to revise number of subjects needed based on initial results of single cell analysis and mtDNA analysis (see Walt Task 3d and Sonenshein Task 4b; months 25-36).

4e. Submit amended IRB proposal adjusting sample size as per 4d (Months 36-42).

4f. Recruit subjects, obtain samples, and provide to Walt lab for single cell analysis studies and to Sonenshein for mtDNA studies (Months 42-60).

KEY RESEARCH ACCOMPLISHMENTS

- Developed assay for measuring EpCAM using digital ELISA with a linear range up to 10 pg/mL (250 fM) and a LOD of 0.88 pg/mL (22 fM) with a coefficient of variation of < 20%.
- Performed successful ligation in high concentration in bulk solution for 100 μM of synthetic target miRNA-21 and DNA to validate ligation process.
- Minimized background by successfully coupling PEG modified DNA to microspheres and achieving a LOD of 2.8 fM for the direct hybridization of short biotinylated miRNA mimics.
- Demonsstrated approximate LOD of 1.3-2 pM for ligation miRNA SiMoA assay using miRNA mimics.
- Performed successful bulk microtiter plate assays for sandwich miRNA SiMoA with LOD of 1.8 pM.

- Established feasibility of direct hybridization and sandwich hybridization experiments with LODs of ~4 fM at room temperature and ~2.5 fM at 60°C using short DNA probes from the gene sequence for GAPDH.
- Developed microfluidic platform to isolate and label CTCs.
- Developed fibroblast cell lines for HIM model
- Established tumor cell implantation in the HIM model in 64 mice to date and are currently monitoring for tumor development.
- Identified primers to sequence the human and mouse mtDNA genome.
- Obtained the sequence of mtDNA in 11 breast cancer cell lines, and identified specific mutations.
- Can detect mtDNA in the blood of mice carrying MDA-MB-231 cell derived tumors and obtain DNA sequence.
- Identified 36 miRNAs that are targets of ADAM8 in MDA-MB-231 cells.
- Obtained terminal bleeds from HIM model and tested samples using digital ELISA assays.
-

REPORTABLE OUTCOMES

- Vu T.L, Geldart Flashman J, Walt, D.R. Development of Digital ELISA for Single Molecule Detection of Breast Cancer Biomarkers. Poster session presented at: 95th Canadian Chemistry Conference and Exhibition; 2012 May 26-30; Calgary, AB

OVERALL SUMMARY AND CONCLUSION

In summary, the efforts carried out towards developing assays to measure four biomarkers using digital ELISA for application in HIM models have shown remarkable potential, even though assay optimization protocols have yet to be implemented. A digital ELISA assay for EpCAM was successfully developed with a LOD of 0.88 pg/mL (22 fM). Unfortunately, detection of EpCAM in the HIM model terminal bleeds using digital ELISA have proven to be unsuccessful, possibly due to the absence of crucial enzymes that would allow for soluble EpCAM to circulate. In regards to the detection of FGF-9, GATA-3, and SERPIN-E2, further investigation into determining compatible antibody pairs needs to be explored in order to develop sensitive assays to measure these proteins in HIM model serum.

The two different miRNA SiMoA platforms currently being investigated, Ligation miRNA SiMoA and sandwich miRNA SiMoA, both showed promising results towards optimizing the pre-established digital ELISA platform to achieve similar ultrasensitive data using miRNA as targets. The ligation platform has a current LOD of ~1.3-2 pM using miRNA mimic sequences, but requires further optimization to reduce background, increase reproducibility, and increase assay efficiency. The addition of PEG spacers to the capture probes in RNA direct hybridization experiments seemed to aid in lowering the background. Future ligation experiments will further investigate the effects of adding PEG-DNA probes. The sandwich miRNA platform demonstrated successful bulk experiments followed by DNA direct hybridizations and sandwich assays at the single molecule level reaching LODs down to ~2.5 fM. Future work on this assay includes optimization to increase assay efficiency and decrease the background baseline as well as to use RNA targets.

Circulating tumor cell isolation using microfluidics has been accomplished and these methods will soon be integrated with the single molecule detection approach,

Good progress on identifying additional markers (miRNA, mtDNA, ADAM8) has been made. Further work to validate whether these markers are useful for detecting early stage BC or tumor aggressiveness is underway.

HIM model studies have been initiated with blood being collected and tested by the Walt Laboratory. Additional cell lines are being developed.

Patient protocol and IRB approvals are in place to initiate prospective studies for validating the biomarker assays once suitable candidate biomarkers have been identified.

REFERENCES

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SUPPORTING DATA

Name	Sequence
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
cp-Ligate	5Phos/TTTTTTTTTTTTTTTTTTTTTTTT/3AmMC6T
ssRNA1	CUCACACUAACGACGGGGAAUUUU
sp-ssRNA	TTCCCCGTCGTTAGTGTGAG/3Bio
b-RNA	5Biosg/CUCACACUAACGACGGGGAAUUUU
cp-DNA	TTCCCCGTCGTTAGTGTGAGTTTTTTTTTT/3AmMC6T
cp-PEG	TTCCCCGTCGTTAGTGTGAG/iSp18/TTTTTTTTTT/AmMC6T
cp-LNA	T+TCCC+CGTCG+TTAG+TGTG+AGTTT/3AmMO
miRNA-21 DNA	TAGCTTATCAGACTGATGTTGA
cp-miRNA-21	5AmMC12/TTTTTTTTTTTCAACATCAGT
sp-miRNA-21	5'-CTGATAAGCTA/3Bio
DH-GAPDH	5/Biosg/CCCACATGGCCTCCAAGGAGTAA
GAPDH-sand	GAAAGGTGAAGGTCGGAGTCAACGGATTTGGTCGTCATGGCCCACATGGCCTCCAAGGAGTAAGA
cp-GAPDH	5/AmMC6/TTACTCCTTGGAGGCCATGTGGG
sp-GAPDH	CCGTTGACTCCGACCTTCACCTT/3Bio

Table S1. DNA and RNA sequences used for all ligation and sandwich miRNA SiMoA assays as described in the Statement of Work, Task 1.

+ denotes the location of a LNA within the sequence